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Technical  
Bulletin No. 1902

# **International Response to Infectious Salmon Anemia: Prevention, Control, and Eradication**



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# **International Response to Infectious Salmon Anemia: Prevention, Control, and Eradication**

Proceedings of a Symposium  
New Orleans, LA  
September 3–4, 2002

Otis Miller, D.V.M., M.S., and  
Rocco C. Cipriano, Ph.D.,  
Technical Coordinators<sup>1</sup>

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## Letter of Transmittal

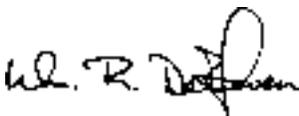
The suppression of infectious salmon anemia (ISA) is key to protecting aquaculture, the fastest growing segment of the U.S. agricultural economy. Government stewards of the salmon resource of the North Atlantic must communicate and cooperate to eradicate ISA before it becomes endemic. Sharing the science already learned by researchers in Scandinavia and the United Kingdom is essential to developing an effective management strategy.

Secretary of Agriculture Ann Veneman acknowledged the importance of ISA when she authorized the expenditure of \$8 million in fiscal years 2002 and 2003 to control and eradicate the disease. APHIS received pass-through funding for ISA research and control efforts in December 2001 and, in spring 2002, put into place program standards to eradicate the disease.

In September 2002, the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) hosted a symposium on ISA with assistance from the U.S. Department of the Interior's U.S. Geological Survey and the U.S. Department of Commerce's National Marine Fisheries Service. The American Veterinary Medical Association supplied organizational expertise as well. This 2-day event was held in connection with the International Symposium on Aquatic Animal Health in New Orleans. These symposium proceedings capture not only the formal scientific presentations but also a court reporter's transcript of the informal presentations at the end of the second day, which addressed real-world considerations for ISA prevention, control, and eradication.

Given the importance of the information shared at the meeting, APHIS staff members have pulled out all the stops to produce this book in 8 months. Once our supply of free copies is exhausted, copies can be purchased from the U.S. Department of Commerce's National Technical Information Service, 5285 Port Royal Road, Springfield, VA 22161. Also, a .pdf version will be placed on the Web site of APHIS' Veterinary Services at <<http://www.aphis.usda.gov/vs/aquaculture>>.

I hope you find the information presented at the conference as useful as I did.



W. Ron DeHaven  
Deputy Administrator, Veterinary Services  
Animal and Plant Health Inspection Service  
U.S. Department of Agriculture  
Washington, DC

## Foreword

On behalf of Secretary of Agriculture Ann Veneman, APHIS Administrator Bobby Acord, and the Deputy Administrator for Veterinary Services, Ron DeHaven, thanks for your interest in learning more about infectious salmon anemia (ISA). This book documents the scientific papers presented at a 2-day symposium held September 3–4, 2002, in New Orleans, LA, during the weeklong meeting of the International Symposium on Aquatic Animal Health. In addition to 18 formal scientific presentations, we have captured comments from individuals who spoke briefly during an open forum held at the end of day 2 of the symposium. While the formal presentations were submitted in advance and put through rigorous peer review, the forum talks were not vetted or corrected, other than for clarity. We took the precaution of using a local court reporter to capture the forum talks verbatim since those speakers were not required to submit manuscripts.

The meeting itself was structured to provide an international response to ISA using the themes of prevention, control, and eradication. Four moderators introduced the main subject topics and the speakers' presentations in the areas of international applied research response, diagnostic and laboratory response, management response, and regulatory response. Our speakers provided the most current international knowledge on the extent of ISA infection in various countries, including the number of salmon farms affected, depopulation statistics, and related issues such as indemnification, regulation, and management. Also covered were effective and ineffective management procedures, projected outcomes of procedures in current use, and new developments in applied ISA science and research, including diagnostics and prevention.

Presenters represented five countries: Canada, Chile, Norway, Scotland, and the United States. When the number of speakers reached 19, we split the meeting into 2 sessions on successive days. The second day culminated in a panel discussion entitled Practical Future Considerations for ISA Prevention, Control, and Eradication. This open forum was designed to allow audience participation and permit ISA experts not on the official agenda to address topics of interest.

It always takes a team of workers to organize and execute a meeting like ours and to bring its results to printed form afterward. We will give you more particulars on this process and identify all the major players in the Acknowledgments section immediately following this Foreword.

Should you wish to contact any speakers or forum participants for more details, appendixes 1 and 2 provide complete contact information.

We look forward to working with you in helping the aquaculture industries and natural-resource agencies manage ISA. The symposium held in New Orleans was an important national and international step in coming to grips with this significant aquatic animal health threat.

A handwritten signature in black ink that reads "Otis Miller, Jr." in a cursive style.

Otis Miller, Jr., D.V.M., M.S.  
National Aquaculture Coordinator  
Chair, ISA Symposium Planning Committee  
USDA-APHIS-Veterinary Services  
Riverdale, MD

A handwritten signature in black ink that reads "Rocco C. Cipriano" in a cursive style.

Rocco C. Cipriano  
Senior Research Microbiologist  
U.S. Department of the Interior  
U.S. Geological Survey  
National Fish Health Research Laboratory  
Kearneysville, WV

## Acknowledgments

The Veterinary Services unit of USDA's APHIS, the U.S. Geological Survey, the National Marine Fisheries Service, and Interior's U.S. Fish and Wildlife Service at the Federal level—along with Maine's Department of Marine Resources at the State level—are hard at work to ensure a successful prevention, control, and eradication program to manage infectious salmon anemia (ISA) in the United States. Similar efforts are being mounted in Canada, Scotland, and Norway, all of which suffered ISA outbreaks before the disease came to U.S. waters.

APHIS supports an integrated approach that uses the expertise of all relevant Federal agencies, States, and Canadian Provinces, as well as industry stakeholders in an international partnership for development and implementation of programs involving aquaculture. Because ISA represents a disease threat to the salmon industry on both sides of the Atlantic, integrating the scientific and regulatory response to this disease across national borders is extremely important. The creation of the first international symposium on ISA held in the United States is an initial step in acknowledging that this disease is everybody's issue. We [Otis Miller and Rocco Cipriano] organized the symposium but not in a vacuum. We gratefully acknowledge the expertise and cooperation of the Office International des Epizooties (Paris), the American Veterinary Medical Association (headquartered in Schaumburg, IL), the Canada Department of Fisheries and Oceans, the Norwegian Animal Health Authority, Scotland's famous marine research lab in Aberdeen, aquaculture units in the Provincial Governments of New Brunswick and Prince Edward Island, and the Maine Aquaculture Association in supplying experts for the podium.

Rocco Cipriano provided yeoman service in the summer of 2002 in setting up a peer-review process for all the manuscripts formally presented. Keeping track of the peer reviewers' comments and making sure that speakers took those comments into account in revising their contributions before the meeting in September was a gargantuan task. Some readers may not understand that Rocco's role as a "Technical Coordinator" for the book far exceeds that of an editor. He also served as the official reviewer of the entire text for the U.S. Geological Survey. We could not have made this proceedings without his help before, during, and after the meeting itself.

The following individuals also worked on putting together the program:

- Peter Merrill, D.V.M., MicroTechnologies, Inc., Richmond, ME
- Jill Rolland, fisheries biologist with USDA-APHIS-Veterinary Services, Riverdale, MD
- Alasdair McVicar, Ph.D., DFO, Aquaculture and Fish Health, Ottawa, ON

- David Scarfe, D.V.M., Ph.D., assistant director of scientific activities with the American Veterinary Medical Association, Schaumburg, IL
- Kevin Amos, Ph.D., national fish health coordinator for the U.S. Department of Commerce's National Marine Fisheries Service, Olympia, WA
- Jim Winton, Ph.D., chief of the fish health section at the U.S. Geological Survey's Western Fisheries Research Center, Seattle, WA
- Gilles Olivier, Ph.D., Canada Department of Fisheries and Oceans, Ottawa, ON

The following individuals helped by volunteering to act as moderators for the four subsections of the program:

- David Scarfe, D.V.M., Ph.D.
- Laura Brown, Ph.D., group leader of genome sciences, National Research Council of Canada, Halifax, NS
- Paul J. Midtlyng, D.V.M., Ph.D., VESO, Oslo, NO
- Carey Cunningham, Ph.D., Fisheries Research Services Marine Laboratory, Aberdeen, UK
- Patricia Barbash, fishery biologist, USDI U.S. Fish and Wildlife Service, Northeast Fisheries Research Center, Lamar, PA

Patricia Barbash and Kevin Amos also provided interagency review of the entire text outside USDA.

Behind the scenes, the following individuals worked on preparing the book for publication:

- Janet S. Wintermute, writer/editor, USDA–APHIS–Legislative and Public Affairs, Riverdale, MD
- Heather Curlett, designer, USDA Design Division, Beltsville, MD
- Jill Rolland and Bronte Williams, manuscript traffic and preparation, USDA–APHIS–Veterinary Services, Riverdale, MD
- Anita McGrady, printing specialist, USDA–APHIS–Legislative and Public Affairs, Washington, DC

Their single-minded dedication to this project is the reason APHIS was able to issue the proceedings in 8 months.

In closing, the fourth International Symposium on Aquatic Animal Health was a highly appropriate setting for a symposium on ISA. If it were not for the unselfish efforts of the organizer of the symposium on aquatic animal health, Dr. Ron Thune, as well as the program chairs, Drs. John Hawke and Jerome La Peyre, APHIS could not have held this meeting.

And since money drives all endeavors, and bookmaking is no exception, now is the time to acknowledge financial contributions from APHIS, the National Marine Fisheries Service, and the U.S. Geological Survey.

I hope you will find that this book enhances your understanding of ISA and supports you in your commitment to help the scientific community deal with it.

— Otis Miller, Jr., D.V.M., M.S.  
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# Infectious Salmon Anemia: The Current State of Our Knowledge

Rocco C. Cipriano and Otis Miller, Jr.<sup>1</sup>

**Abstract:** Infectious salmon anemia (ISA) is a highly infectious viral disease that causes acute mortality principally among Atlantic salmon (*Salmo salar*). The cause of ISA is an orthomyxolike enveloped virus that replicates throughout most host tissues, including midkidney, head kidney, liver, spleen, intestine, gills, muscle, and heart. The virus is cultured in Atlantic salmon head kidney (SHK-1) cells, in the Chinook salmon (*Oncorhynchus tshawytscha*) embryo (CHSE-214) cell line, and within the TO cell line developed from head kidney leucocytes. Clinical signs of the disease may include pale gills, ascites, liver congestion, enlarged spleen, petechial hemorrhages within visceral fat, congestion of the gut, and severe anemia. The disease is pronounced in the marine environment, where it is most often transmitted by cohabitation with infected live salmon, infected biological materials, or contaminated equipment. Control of ship and personnel movements among infected sites, destruction of infected lots, and the closure and fallowing of virus-contaminated areas may be used to reduce the likelihood of further spread of the disease.

## Hosts and Geographic Range

Infectious salmon anemia (ISA) is a highly infectious disease of Atlantic salmon (*Salmo salar*) that was first reported in Norwegian aquaculture facilities. The disease has since been described among premarket Atlantic salmon in Scotland (Bricknell et al. 1998), New Brunswick, Canada (Lovely et al. 1999, Jones et al. 1999a), the United Kingdom (Rodger et al. 1999), the Cobscook Bay region of Maine (Bouchard et al. 2001), and in the Faroe Islands (Anonymous 2000). The virus that causes ISA has also been detected among coho salmon (*Oncorhynchus kisutch*) in Chile (Kibenge et al. 2001). In Canada, the disease was first characterized as a new condition termed "hemorrhagic kidney syndrome" or HKS (Byrne et al. 1998). The pathology of HKS was later shown to be caused by ISAv (Bouchard et al. 1999, Lovely et al. 1999), although laboratory confirmation of ISA virus (ISAv) was initially complicated by dual isolation of that virus and a nonpathogenic Toga-like virus from HKS samples (Kibenge et al. 2000a).

The rapid invasion of ISAv into three bays within New Brunswick and its subsequent spread among 21

farms (Bouchard et al. 1998) indicate the severe nature of the threat that ISA represents for Atlantic salmon aquaculture. Furthermore, the annual cost of ISA outbreaks among farmed fish in 1999 was reported, in U.S. dollars, to be \$11 million in Norway and \$14 million in Canada. The 1998–99 epidemics in Scotland were valued at a cost of \$32 million (Hastings et al. 1999). Although epizootics of ISA have been specifically associated with cultured salmon (Department of Fisheries and Oceans [DFO]—Canada), biologists have also detected the presence of ISAv among Atlantic salmon populations that are wild or have escaped from aquaculture operations at the Magaguadavic River fish trap (Bay of Fundy, NB). In addition to Atlantic and Chinook salmon, the pathogen infects, but has not produced disease in, freshwater brown trout (*Salmo trutta*) (Nylund et al. 1995a), sea trout (*S. trutta*) (Nylund and Jakobsen 1995), and rainbow trout (*Oncorhynchus mykiss*) (Nylund et al. 1997). Although the virus has been detected in saithe (*Pollachius virens*), it is unable to replicate in such hosts (Raynard et al. 2001).

## Etiology

The cause of ISA is an enveloped virus 45–140 nm in diameter (Dannevig et al. 1995b) with a buoyant density 1.18 g/mL in sucrose and cesium chloride gradients. It shows maximum replication at 15 °C but strongly reduced replication at 25 °C (Falk et al. 1997). The virus may be cultured in the SHK-1 cell derived from Atlantic salmon pronephros cells and produces variable cytopathic effects (CPE) between 3 and 12 days after inoculation (Dannevig et al. 1995a,b; Kibenge et al. 2000b). The ISAv also replicates and produces CPE within the Atlantic salmon head kidney (ASK) cell line developed by Devold et al. (2000) and the TO cell line developed from Atlantic salmon head kidney leukocytes by Wergeland and Jakobsen (2001). Some, but not all, strains of ISAv will also replicate in Chinook salmon (*Oncorhynchus tshawytscha*) embryo (CHSE-214) cells and produce CPE between 4 and 17 days after inoculation (Kibenge et al. 2000b). The virus also

<sup>1</sup> Dr. Cipriano is with the U.S. Geological Survey's National Fish Health Research Laboratory in Kearneysville, WV. Dr. Miller is with USDA-APHIS' Veterinary Services in Riverdale, MD.

replicates within, but does not produce distinct CPE in, the AS cell line (Sommer and Mennen 1997). Growth is inhibited by actinomycin D but not by 5-bromo-2-deoxyuridine (Sommer and Mennen 1997, Falk et al. 1997), and the virus is most closely related to other orthomyxoviruses (Mjaaland et al. 1997, Krossøy et al. 1999, Sandvik et al. 2000). Four major polypeptides are evident with estimated molecular sizes of 71, 53, 43, and 24 kDa (Falk et al. 1997).

Mjaaland et al. (1997) indicated that the total genome of ISAv (14.5 kb) consisted of eight segments between 1 and 2.3 kb. This genetic analysis suggests a close relationship between ISAv and other viruses in the family *Orthomyxoviridae*, but the smallest genomic segment (segment 8) is not homologous with any other known sequence data. Development of a primer set from this segment therefore had significant diagnostic value. Krossøy et al. (1999) further established a relationship between ISAv and other orthomyxoviruses by examining the highly conserved orthomyxovirid PB1 protein encoded by segment 2. Intrafamily genetic comparisons conducted in this manner showed that ISAv has a distant relationship with other orthomyxoviruses and is more closely related to the influenza viruses than to the Thogoto viruses. The relationship to the orthomyxoviruses was further strengthened by the demonstration by Sandvik et al. (2000) that the ISAv genomic segments had conserved 3'- and 5' ends typical for orthomyxoviruses, and the ISAv mRNA has heterologous 5'-ends—indicating a replication strategy more related to the influenza viruses than the Thogoto viruses.

On the basis of the Krossøy team's genetic characterizations and the psychrophilic nature of this virus, which potentially restricts its host range to poikilothermic vertebrates, those authors proposed that ISAv be the type species of a new genus, *Aquaorthomyxovirus*. The name Isavirus has been proposed by the International Committee on Taxonomy of Viruses version 3 (<http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm>) for this genus, in preference to *Aquaorthomyxovirus* suggested by Krossøy et al. (1999).

Nucleotide variations in segments 2 and 8 were used to differentiate Scottish isolates of ISAv from those of Norwegian or North American origins (Cunningham and Snow 2000, Krossøy et al. 2001). Despite such differentiation, the Scottish isolate was more closely related to the Norwegian strain than it was to the North American strain. These results may initially have indicated that geographic proximity influences the ISAv genotype, suggesting that distinct strains occur on both sides of the Atlantic Ocean. However, Ritchie et al. (2001a) showed that nucleotide sequences of an ISAv isolate obtained from Atlantic salmon in Nova Scotia were more similar to Norwegian and Scottish strains than to isolates from neighboring New Brunswick. Although differences were not detected in the nucleotide sequences analyzed, the Nova Scotian isolate did not cause typical ISA and was, therefore, considered to be functionally different from the Scottish and Norwegian isolates. On the basis of calculated evolutionary mutation rates in segment 2, Krossøy et al. (2001) suggested that Canadian and Norwegian ISAv isolates diverged about 100 years ago, which, interestingly enough, coincided with anthropogenic movements of salmonid fishes (particularly via transport of sea trout), between Europe and North America.

The virus possesses hemagglutinating as well as fusion and receptor-destroying activity. The latter activity has been suggested to be caused by an acetylesterase (Falk et al. 1997). Devold et al. (2001) have shown that the hemagglutinin (HA) gene contains a highly polymorphic region (HPR), which shows sequence variation where distinct groups of isolates predominate within certain geographic areas. Additionally, sequence analyses have been provided for segment 3, which encodes for the nucleoprotein (NP) that has an approximate mass of 71 to 72 kd (Snow and Cunningham 2001, Ritchie et al. 2001b, Clouthier et al. 2002); for segment 4, which is purported to be a polymerase encoding gene for the P2 protein (Ritchie et al. 2001b, Clouthier et al. 2002); for segment 6, which encodes a 38–42-kd glycosylated protein determined to be the HA analogue (Griffiths et al. 2001, Clouthier et al. 2002, Kibenge et

al. 2002, Devold et al. 2001); and for segment 7, which possibly encodes for two matrix genes expressing the P4/P5 proteins (Biering et al. 2002). Clouthier et al. (2002) have completed a genetic map for ISAv and, in addition to what has been noted above, these authors have completed analysis of segments 1 and 5. Segment 1 has been shown to encode for the P1 protein and segment 5, for the 47-kd glycosylated P3 protein suggested to be a surface glycoprotein.

## Pathogenicity

Considerable viral replication occurs within infected fish, and the virus may become widely disseminated throughout most tissues, including midkidney, head kidney, liver, spleen, intestine, gills, muscle, and heart (Jones et al. 1999a, Rimstad et al. 1999). Dannevig et al. (1994) suggested that liver cells, leukocytes, and immature erythrocytes are target cells for replication of ISAv. Further study by Nylund et al. (1996) supported the hypothesis that leukocytes may be target cells for ISAv and showed that it can actually replicate in endothelial cells lining the blood vessels in the ventricle of the heart, in endocardial cells, and in polymorphonuclear leukocytes. In tissue culture, the virus binds to sialic acid residues on the cell surface and fuses with endosomes and liposomes, where binding, uptake, and fusion are enhanced as pH values are decreased from 7.5 to 4.5 (Eliassen et al. 2000).

Clinical signs may be evident 2–4 weeks following infection and commonly include pale gills, ascites, enlargement of the liver and spleen, petechiae in the visceral fat, congestion of the gut, severe anemia, and mortality (Hovland et al. 1994, Thorud and Djupvik 1988, Evensen et al. 1991). Microscopic pathological changes are commonly characterized by renal interstitial hemorrhage and tubular necrosis, branchial lamellar and filamental congestion, congestion of the intestine and pyloric caecae, and perivascular inflammation in the liver (Mullins et al. 1998b, Rimstad et al. 1999). Prominent lesions are often reported in the parenchyma and vascular system of the liver, where congestion and

degeneration of hepatocytes are often followed by hemorrhagic necrosis in the latter stages of disease (Evensen et al. 1991).

Speilberg et al. (1995) concluded that lesions in the liver may not be the sole result of an anemia because significant ultrastructural damage had already occurred before a decrease in hematocrit values and any viral-induced disruption of the hepatocytes had been observed. These observations suggest that the lesions in the liver may result from an impeded sinusoidal blood flow that culminates in an ischemic hepatocellular necrosis (Speilberg et al. 1995). Decreases in hepatic glutathione of up to 70 percent observed in diseased fish may affect the capability of the liver to transform and excrete xenobiotics from the body (Hjeltnes et al. 1992).

The development of anemia suggests that erythrocytes may be among the most important target cells of the virus. Anemia often develops rather late in the course of infection (Dannevig et al. 1994), and a leukopenia is suggested to develop concomitantly with the anemia (Thorud 1991). However, Dannevig's team demonstrated that head kidney leukocytes are infected earlier and are probably more important than erythrocytes in replication of the virus earlier in the infective process. Still, fish injected with the ISAv may display a suppressed leukocyte response that does not necessarily correlate with the development of erythrocytic anemia (Dannevig et al. 1994). Suppression of immune function and development of anemia, therefore, appear to be independent events.

Experimentally, elevated plasma cortisol concentrations have been correlated to the severity of anemia as measured by hematocrit values (Olsen et al. 1992). Plasma lactate may also be elevated in diseased fish (Olsen et al. 1992).

## Transmission

The disease is pronounced in the marine environment, where it is most often transmitted by cohabitation with infected live salmon or infected biological materials such as animal wastes or

discharges from normal aquaculture operations, slaughter facilities (Vagsholm et al. 1994), and contaminated wellboats (Shannon 1998, Murray et al. 2002). Infected fish may transmit the disease weeks before they show apparent signs of infection. The virus may spread horizontally, from fish to fish, by shedding of virions from the blood, gut contents, urine, and epidermal mucus of infected salmon (Totland et al. 1996). Moreover, fish that survive epizootics may shed viral particles for more than 1 month into the surrounding water (Hjeltnes et al. 1994), within which the virus is relatively shortlived and may persist for only about 20 hours at 6 °C and up to 4 days in tissues at the same temperature (Nylund et al. 1994b). Consequently, infected biological materials, such as animal wastes or discharges from aquaculture operations, slaughter facilities (Vagsholm et al. 1994), and contaminated wellboats (Shannon 1998, Murray et al. 2002), may establish better reservoirs of infection than the water column alone. Blood and mucus contain especially large amounts of virus and more effectively transmit the disease than feces, plankton, and salmon lice (Rolland and Nylund 1998).

Sea lice of the species *Caligus elongatus* and *Lepeophtheirus salmonis*, however, may also be important vectors of the virus during epidemics (Nyland et al. 1994b). There is no evidence that scallops cocultured with Atlantic salmon either accumulate the pathogen or transmit the disease (Bjoershol et al. 1999). The pathogen can be transmitted to, but has not produced disease in, freshwater brown trout (Nylund et al. 1995a), sea trout (Nylund and Jakobsen 1995), sea-run brown trout (Rolland and Nylund 1999), and rainbow trout (Nylund et al. 1997), suggesting that these fish may become carriers and serve as potential reservoirs of infection (Nylund et al. 1997). Although brown trout produce neutralizing antibodies against ISAv within 45 days after primary infection, the virus may still be present 7 months after infection (Nylund et al. 1994a).

Clearance of the virus following experimental infection progresses at a greater rate in Arctic char

(*Salvelinus alpinus*) than in rainbow trout and brown trout. Thus, the potential for char to act as a long-term carrier of ISAv may be less than that of other salmonids, all of which apparently clear viable virus by 40 days following injection (Snow et al. 2001).

Horizontal transmission of ISAv in fresh water has been achieved experimentally (Brown et al. 1998) and occurs rapidly between infected and naive smolts in fresh water. Even under these conditions, asymptomatic smolts may remain infective to naive parr for 18 months after the original challenge (Melville and Griffiths 1999).

Vertical transmission of the virus from parent to offspring via intraovum infection has not been demonstrated (Melville and Griffiths 1999). Even though it is commonly believed that the virus is not transmitted vertically, ISAv mortality has been reported among first-feeding fry (Nylund et al. 1999). This scenario emphasizes the importance of screening brood fish and conducting proper egg disinfection procedures to reduce contagion in early life stages.

## **Diagnostics and Detection**

Viral replication and the development of CPE in tissue culture are routinely used as the standard by which all other diagnostic and detection assays are measured. As already mentioned, the virus can be cultured in Atlantic SHK-1 cells, the CHSE-214 cell line, the ASK cell line, the TO cell line, and the AS cell line. The focal CPE associated with ISAv growth in CHSE-214 cells suggest that this cell line could provide the foundation for a culture-based diagnostic. The lack of focal CPE has been viewed as a disadvantage associated with SHK-1 and AS cells. Unfortunately, the CHSE-214 line does not support the growth of all ISAv isolates (Kibenge et al. 2000b). The inability of some ISAv isolates to replicate in CHSE-214 cells and the lack of distinct CPE in either AS or SHK-1 complicate effective and consistent cultural detection of this virus. Consequently, parallel use of both SHK-1 or CHSE-214 cells provides more sensitive detection of ISAv than use of either

cell line alone (Opitz et al. 2000). Further development and greater availability of the ASK and TO cell lines may alleviate these problems.

Nonculture-based diagnostics that detect ISAv include an indirect fluorescent antibody test (Falk and Dannevig 1995a) and a reverse-transcriptase–polymerase chain reaction (RT–PCR) procedure (Mjaaland et al. 1997). Further confirmation has been effected by the use of a DNA probe employing primer sets developed against segment 8 of the virus (McBeath et al. 2000).

Devold et al. (2000) found RT–PCR to be more sensitive for detection of ISAv among carrier sea trout than either culture or injection of suspect blood samples into naïve fish. Furthermore, RT–PCR screens of gill mucus present an accurate and sensitive nonlethal alternative for detection of the virus from other tissues that require necropsy (Griffiths and Melville 2000).

Production of a monoclonal antibody against ISAv enabled Falk et al. (1998) to conduct several serodiagnostic assays, including the enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody staining of virus-infected cell cultures, immunoelectron microscopy of negatively stained virus preparations, virus neutralization assay, and hemagglutination inhibition assay.

Atlantic salmon that survive infections with ISAv (Falk and Dannevig 1995b) or are either passively (Falk and Dannevig 1995b) or actively immunized (Brown et al. 2001, Jones et al. 1999b) against the virus develop an immune resistance against this pathogen. Recently, an indirect ELISA assay was developed to detect antibodies to ISAv in Atlantic salmon sera (Kibenge et al. 2002). In a diagnostic sense, this assay can theoretically denote previous exposure to the pathogen among nonvaccinated fish by detection of viral-specific antibodies. The assay also permits titration of ISAv-specific antibodies as a consequence of vaccination. In general, the current nonculture-based methods for routine detection and confirmation of ISAv, in decreasing order of sensitivity and specificity, are RT–PCR, antibody ELISA, immunofluorescence, and histopathologic

examination (Groman et al. 2001). It is important to note that the phenotypic (Kibenge et al. 2000b) and genomic (Blake et al. 1999) differences that exist among ISAv isolates may influence the use of specific assays.

## Management

Because of the acute nature of the disease and an inability to control mortality, European Economic Community countries require compulsory slaughter of infected stocks (Hill 1994). Similar eradication programs have been enacted in Canada (Mullins 1998a). Because the virus is readily transmitted in seawater, such dissemination may readily contaminate culture facilities within 5 to 6 km of an infected site within a 6- to 12-month period (Eide 1992). It is recommended, therefore, that culture sites be spaced no less than 5–6 km apart and that wastewater from slaughter and processing facilities be thoroughly disinfected (Jarp and Karlsen 1997).

Further contagion may be managed by control of ship and personnel movements among sites, destruction of infected lots, and the closure and following of contaminated sites (Murray 2001). Iodophor, chloramine–T, and chlorine dioxide have been shown to be effective topical disinfectants against ISAv when used for a minimum of 5 minutes according to manufacturer's instructions (Smail et al. 2001).

Nylund et al. (1995b) have observed greater overall resistance to ISA among two wild strains of Atlantic salmon than was noted in a strain used in commercial aquaculture. Because all other physical parameters that may have affected challenge results were held constant, the observed patterns of resistance were believed to result from genetic differences among the strains. Such differences could theoretically be used to select for resistance to ISAv (Dahle et al. 1996). The management of ISAv through the development of disease-resistant strains of fish, however, is not consistent with current control practices that involve destruction of infected populations and disinfection of contaminated sites.

## Vaccination

Jones et al. (1999b) have shown that vaccination via intraperitoneal injection of inactivated virus elicited the best protection if at least 734 degree-days had elapsed between vaccination and challenge. These studies resulted in the development of a commercially licensed autogenous product used within specific areas (McDougall et al. 2001). Protection was significantly improved if the viral antigen was delivered in an oil emulsion (Jones et al. 1999b, Brown et al. 2001). Christie et al. (2001) also indicated that vaccination may produce relative percent survival values of 90 or higher (54 percent mortality among controls) for 6 months after vaccination without significant risk of viral transmission by vaccinated fish that may have become asymptomatic carriers. The latter research was the result of a consortium of scientists from the University of Bergen, the National Veterinary Institute, and Intervet Norbio and was supported financially by the Norwegian Research Council. A multivalent vaccine including ISAV, infectious pancreatic necrosis virus, *Vibrio anguillarum* (two serotypes), *V. salmonicida*, *Aeromonas salmonicida*, and *Moritella viscosa* prepared in a water in oil formulation is projected for commercial availability within Norwegian and Faroe Island markets by early 2003. Multivalent vaccine combinations designed for the Canadian and United Kingdom markets are also in development.

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# Role and Function of the OIE Fish Diseases Commission in the Field of Aquatic Animal Health

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**Abstract:** The Office International des Epizooties (OIE) is the World Organization for Animal Health; it currently comprises 162 member countries. While representation is usually through the member countries' chief veterinary officers, competent authorities other than the national veterinary services may be responsible for aquatic animal health in some OIE member countries.

In 1960, the OIE established the Fish Diseases Commission (FDC) because of increasing awareness of the importance of international trade in fish and other aquatic animals. In 1988, the scope of the FDC was extended to include diseases and pathogens of molluscs and crustaceans.

The expansion of international trade in aquatic animals and their products has called for appropriate rules to avoid the risk of spread of communicable diseases.

## Introduction

The Office International des Epizooties (OIE, World Animal Health Organization)—an intergovernmental organization headquartered in Paris—was created by an international agreement on January 25, 1924, signed in Paris by 28 countries. In May 2002, the OIE totaled 162 member countries worldwide. Representation in OIE is through national delegates, usually the chief veterinary officer of the country. However, competent authorities other than the national veterinary services may be responsible for aquatic animal health in some member countries, and this fact makes it necessary for the veterinary administrations and other competent authorities to cooperate for the benefit of aquatic animal health.

## Structure of the OIE

Each OIE member country appoints a delegate. All the delegates form the OIE International Committee, which meets once a year in Paris to hold its general session. The International Committee is the highest authority within the OIE.

At the general session, the member countries elect the president of the International Committee, as

Standardization of aquatic animal health requirements for trade and harmonization of international aquatic animal health regulations are critically important to enable trade to continue while maintaining effective national disease control. The international aquatic animal health standards prepared by the FDC are laid down in two important documents, OIE International Aquatic Animal Health Code and the Diagnostic Manual for Aquatic Animal Diseases. Currently, the Code and Manual specifically deal with 13 “diseases notifiable to the OIE” and 18 “other significant diseases” of aquatic animals. The diseases are classified into one of these two lists on the basis of their socio-economic importance, geographic range and etiology. Infectious salmon anemia (ISA) is currently listed under the list of “other significant diseases,” and both the Code and Manual have chapters dealing with the disease.

well as members of the administrative, regional, and specialist commissions. All these positions are held for 3 years.

The specialist commissions are composed of members elected by the International Committee and are either OIE delegates or internationally renowned experts from OIE member countries.

Currently, the OIE has four specialist commissions:

- The Foot and Mouth Disease and Other Epizootics Commission (created 1946),
- The Standards Commission (1949),
- The International Animal Health Code Commission (1960), and
- The Fish Diseases Commission (FDC) (1960).

The FDC was established to deal specifically with the increase in fish diseases as aquaculture expanded worldwide. As of 1988, the scope of the FDC was extended to include diseases and pathogens of molluscs and crustaceans as well. The FDC has five members.

The OIE has these main objectives:

- To ensure transparency in the animal health situation throughout the world, including aquatic animal health;
- To collect, analyze, and disseminate scientific veterinary information;

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- To contribute expertise and encourage international coordination in the control of animal diseases, including aquatic animal diseases;
- Within its mandate under the Agreement on Sanitary and Phytosanitary (SPS) measures of the World Trade Organization (WTO), to safeguard world trade by publishing health standards for international trade in animals and animal products; and
- To improve the legal framework and resources of veterinary services in the member countries.

The principal policy of the OIE is to facilitate international trade in animals and animal products, including aquatic animals and their products, based on health control and preventative measures. The scope also covers food safety and animal welfare.

Over the years, the OIE had and still has an important role to play in establishing a framework that may be used for strategic planning and decisionmaking in OIE member countries.

## **Publication of Standards**

The expansion of international trade in animals and animal products including aquatic animals since the 1960s has called for:

- Appropriate veterinary regulations to avoid the risk of communicable diseases spreading to animals or even to humans;
- Standardization of animal health requirements applicable to trade, to avoid unnecessary hindrances; and
- Harmonization of international animal health regulations. (This is critically important to ensure growth in international trade while maintaining effective national disease control.)

The International Aquatic Animal Health Code (OIE 2002a) (henceforth referred to as the Aquatic Code) drawn up by the FDC meets these requirements. Provisions are given as guidelines for the preparation of veterinary regulations for import and export. This regularly updated collection of recommended veterinary requirements for

international trade takes into account special conditions prevailing in various countries and offers appropriate solutions for each one.

The Aquatic Code, which was approved for the first time by the International Committee in May 1995 (Håstein 1996), currently covers a list of 13 “diseases notifiable to the OIE” and a list of 18 “other significant diseases” of aquatic animals of which the international community needs to be aware. Diseases have been classified into one of these two lists on the basis of their socioeconomic importance, geographic range, and etiology.

Prior to 1995, when the first edition of the Aquatic Code and the Diagnostic Manual for Aquatic Animal Diseases (OIE 2000) (henceforth referred to as the Diagnostic Manual) were adopted by the OIE International Committee, aquatic animal diseases were included in the OIE International Animal Health Code (OIE 2002b), which covers diseases of terrestrial animals. This way of providing recommendations for sanitary measures to be applied to aquatic animals held obvious drawbacks. A decision to publish a separate Code and Manual for aquatic animals was thus initially taken in the late 1980s. The FDC carried out the onerous work of preparing both texts with a view to producing a separate set of documents based on the same template as that for terrestrial animals, but bearing in mind the specific criteria needed for aquatic animals. In addition to material provided by members of the FDC, contributions have also been made by the OIE International Animal Health Code Commission, the OIE Standards Commission, and scientific experts in various OIE member countries. Assistance was provided through comments and information needed as well as through preparation of certain chapters on diseases for which the FDC members themselves did not possess the necessary expertise. The end result is thus based on international teamwork.

Not all countries will be able to comply with each of the specifications detailed in the Aquatic Code and Diagnostic Manual; probably only a few countries will have the necessary resources. In addition, many countries may still have concerns that

they would like to see resolved before subscribing to all the principles given in the Aquatic Code, namely, zoning principles, health certification, etc.

The Aquatic Code sets out general principles covering:

- Definitions,
- Notification systems,
- Veterinary ethics and certification for international trade,
- Import risk analysis,
- Import/export procedures,
- Contingency plans,
- Diseases notifiable to the OIE (fish, molluscs, and crustaceans),
- Other significant diseases (fish, molluscs, and crustaceans),
- Health control and hygiene, and
- Model international aquatic animal health certificates approved by the OIE.

Currently, the FDC is preparing a chapter on fallowing of aquaculture enterprises. This chapter has not yet been approved by the OIE International Committee.

## **Notifications and Epidemiologic Information**

The urgency of dispatching information varies according to the nature of the disease. The OIE has devised a warning system whereby member countries can take action rapidly should the need arise. Countries are required to notify the Central Bureau within 24 hours if there is/are

- A first occurrence or recurrence of a disease notifiable to the OIE if the country or zone was previously considered to be free of that particular disease;
- The emergence of new important findings or a provisional diagnosis of diseases notifiable to the OIE

that are of epidemiologic significance to other countries; or

- New findings (for disease not notifiable to the OIE) that are likely to have exceptional epidemiologic significance to other countries.

The OIE immediately dispatches the incoming data by telex, fax, or electronic mail directly to member countries at risk and in weekly announcements (in Disease Information) to other countries.

In addition to this alert system, information received from member countries is distributed on a periodical basis in the monthly Bulletin, the annual World Animal Health Yearbooks, which provide annual animal health statistics and give data on the occurrence of diseases in each member country and the annual Animal Health Status reports for all OIE member countries.

Although the responsibility for aquatic animal health in many OIE member countries lies with competent authorities other than the veterinary administration, nevertheless, within the OIE system, the veterinary services have the responsibility for disease reporting in conjunction with the competent authority in a given country. Thus, close cooperation between the veterinary services in a given country and the responsible authority for aquatic animal health is strongly needed.

## **Veterinary Ethics and Certification for International Trade**

There may be different philosophies and opinions in regard health certification, ethics, etc., in different countries, but the Aquatic Code describes basic principles that should be taken into account to ensure that trade is unimpeded and that such trade does not constitute a risk to aquatic animal health. Information on the aquatic animal health situation worldwide is thus important in order to diminish the risk of disease transfer through international trade in aquatic animals and their products. Certification must be based on the strictest possible ethical rules.

The chapters in this section of the Aquatic Code present the general requirements and principles of certification to be followed.

### ***Import Risk Analysis***

Any importation of aquatic animals or related products may involve a risk of disease transfer to the importing country. The Aquatic Code chapter on import risk analysis provides an objective and defensible method for assessing risks associated with importation. This basis enables the importing and exporting countries to have productive discussions on problems associated with the potential risks.

### ***Import and Export Procedures***

In the context of import and export, it is important to have general arrangements for transportation of aquatic animals. The chapters in this section of the Aquatic Code provide recommendations for transport and aquatic animal health measures before, during and upon arrival, for frontier posts in importing countries as well as measures concerning international transfer of pathological material and biological products.

### **Contingency Plans**

A number of diseases pose a threat to aquaculture as well as to wild stocks of aquatic animals worldwide; therefore, such diseases may cause significant losses if introduced into countries where they are not established. Bearing this in mind, all disease control should be based on a legislative framework that includes contingency plans. A contingency plan can be defined as an established plan that is designed to have a rational approach for actions to be taken if emergency situations occur and in which all types of required actions should have been considered and described in advance. The Aquatic Code gives guidelines for contingency planning.

## **OIE International Aquatic Animal Health Code: Listing of Pathogens and Diseases**

In the International Animal Health Code, notifiable diseases are divided into Lists A and B on the basis of their seriousness. In the Aquatic Code, as explained before, aquatic animal diseases are classified as either “diseases notifiable to the OIE” or “other significant diseases.”

Diseases and pathogens are included in the Aquatic Code according to the following basic considerations: resistance or response to therapy, geographic range, and socioeconomic importance. The list of diseases and pathogens considered for inclusion in the Aquatic Code is currently restricted to those affecting fish, molluscs, or crustaceans (table 1). Proposals for diseases to be listed may come from member countries or from the FDC, and thorough discussions take place before a new disease is actually added to either list. Similar procedures are followed for deletion of diseases from the lists.

Categorization of diseases to be listed is, of course, open to debate, and opinions on which diseases should be listed vary greatly due to different views on the significance and importance of a given disease. For several years, the FDC has been working to provide a disease categorization system that objectively classifies diseases for listing. At the FDC meeting in June 2002, the Commission proposed a set of criteria suitable for listing aquatic animal diseases, and this has been sent to member countries for comments (table 2). The comments will be considered in relation to the OIE’s work on new procedures for disease notification for terrestrial and aquatic animal diseases. Until the new procedures have been adopted, there will be no changes to the current listed diseases.

Infectious salmon anemia (ISA) was first brought to the attention of the OIE in the early 1990s because there was a need for a common scientific name for the disease. Until then, the disease was referred to by numerous different names in published

papers. The OIE recognized that disease and named it ISA in 1990.

When preparing the first edition of the Aquatic Code, the FDC concluded that in addition to the list of diseases notifiable to the OIE, a “waiting” list should be established for diseases that at a later stage should either be listed as notifiable or should be removed from consideration. ISA was put on this waiting list in 1992 and included in the list of other

**Table 1—Diseases of aquatic animals**

**Diseases Notifiable to the OIE**

**Fish Diseases**

Epizootic hematopoietic necrosis  
Infectious hematopoietic necrosis  
*Oncorhynchus masou* virus disease  
Spring viremia in carp  
Viral hemorrhagic septicemia

**Molluscan Diseases**

Bonamiosis (*Bonamia exitiosus*, *B. ostrea*, *Mikrocytos roughleyi*)  
MSX disease (*Haplosporidium nelsoni*)  
Martelliosis (*Marteilia refringens*, *M. sydneyi*)  
Mikrocytosis (*Mikrocytos mackini*)  
Perkinsosis (*Perkinsus marinus*, *P. olseni/atlanticus*)

**Crustacean Diseases**

Taura syndrome  
White spot disease  
Yellowhead disease

**Other Significant Diseases in Fish, Molluscs, and Crustaceans**

**Fish Diseases**

Channel catfish virus disease  
Infectious pancreatic necrosis  
Infectious salmon anemia  
Viral encephalopathy and retinopathy  
Red sea bream iridoviral disease  
White sturgeon iridoviral disease  
Bacterial kidney disease (*Renibacterium salmoninarum*)  
Enteric septicemia (*Edwardsiella ictaluri*)  
Piscirickettsiosis (*Piscirickettsia salmonis*)  
Epizootic ulcerative syndrome  
Gyrodactylosis (*Gyrodactylus salaris*)

**Molluscan Diseases**

SSO disease (*Haplosporidium costale*)  
Withering syndrome of abalone (*Candidatus Xenohalotis californiensis*)

**Crustacean Diseases**

Crayfish plague (*Aphanomyces astaci*)  
Infectious hypodermal and haematopoietic necrosis  
Spawner-isolated mortality virus  
Spherical baculovirosis (*Penaeus monodon*-type baculovirus)  
Tetrahedral baculovirosis (*Baculovirus penaei*)

significant diseases when that list was created in 1993. Thus, ISA was on the list of other significant diseases in the 1995 edition of the Aquatic Code. ISA was placed on this list and not on the list of diseases notifiable to the OIE because, at that time, ISA was not yet sufficiently defined. Its etiology was not understood well enough, and approved diagnostic methods were not available. On more recent occasions, the question of listing ISA as a notifiable disease has been raised, but it has been decided that no change in listing should be effected until a new notification system for aquatic animal diseases has been approved.

As stated previously, the current notification system requires that new findings of a nonnotifiable disease such as ISA in aquatic animals shall be reported to the OIE immediately if it is of exceptional epidemiologic significance. ISA falls into this category, and occurrence of this disease should be reported to the OIE immediately when ISA is diagnosed in a country for the first time. Table 3 lists the OIE member countries that have reported ISA or ISA virus (ISAv).

**Health Control and Hygiene**

Health control and hygiene prior to international trade in live aquatic animals and their products is also an important issue. This section of the Aquatic Code provides guidelines for hygienic precautions, such as the destruction of pathogens through disinfection procedures in farms producing fish, molluscs, or crustaceans. This section is currently being updated for the next edition of the Aquatic Code. Furthermore, a general chapter on procedures for destruction of carcasses in connection with an outbreak of disease is in preparation.

**Model International Aquatic Animal Health Certificates**

The Aquatic Code contains five different model health certificates that standardize certification paperwork worldwide. The certificates are continuously being improved following comments received from OIE member countries.

**International Response to Infectious Salmon Anemia:  
Prevention, Control, and Eradication**

**Table 2—Proposed criteria for listing and for urgent notification of aquatic animal diseases (June 2002) listing criteria**

	<b>Parameters that support a listing</b>	<b>Explanatory notes</b>
<b>Consequences</b> (any one suffices)	Where it occurs, the disease has been shown to cause significant production losses due to morbidity <sup>1</sup> or mortality on a national or multinational or (zonal regional) level.	The disease generally leads to losses in susceptible <sup>2</sup> species, and morbidity or mortality is related primarily to the agent and not to management or environmental factors.
	The disease has been shown to, or is strongly suspected to, negatively affect wild aquatic animal populations that are an asset worth protecting.	See above.
	The agent is of public health concern.	
<b>Spread</b> (either of the first two plus the third and fourth)	Infectious etiology of the disease is proven.	
	An infectious agent is strongly associated with the disease, but the etiology is not yet known.	Infectious diseases of unknown etiology can have equally high-risk implications as those diseases where the infectious etiology is proven. While disease occurrence data are gathered, research should be conducted to elucidate the etiology of the disease and the results be made available within a reasonable period of time.
	Potential for international spread, including via live animals, their products, and inanimate objects.	Under international trading practices, the entry and establishment of the disease is likely.
	Several countries or zones are free of the disease based on the recommendations of the Code and Manual.	Free countries or zones could still be protected. Listing of diseases that are ubiquitous or extremely widespread would render notification infeasible; however, individual countries that run a control program on such a disease can demand its listing, provided that they have undertaken a scientific evaluation to support their request. Examples may be the protection of broodstock from widespread diseases, or the protection of the last remaining free zones from a widespread disease.
<b>Diagnosis</b>	A repeatable, robust means of detection or diagnosis exists.	A diagnosis test should be widely available, or has undergone a formal standardization and validation process using routine field samples (see OIE Diagnostic Manual for Aquatic Animal Diseases).
<b>Urgent Notification</b>		
<b>Listed diseases</b>		
First occurrence or recurrence of a disease in a country or zone of a country if the country or zone of the country was previously considered to be free of that particular disease		
Occurrence in a new host species		
New pathogen strains or new disease manifestation		
Potential for international spread of the disease		
Zoonotic potential		
<b>Nonlisted diseases</b>		
Emerging disease or pathogenic agent if there are findings that are of epidemiologic significance to other countries		

<sup>1</sup>“Morbidity” includes, for example, loss of production due to spawning failure.

<sup>2</sup> “Susceptible” is not restricted to “susceptible to clinical disease” but includes “susceptible to covert infections.”

**Table 3—Countries having reported the detection of ISA and/or ISA virus**

Country	Year of first detection	Region	Species
Canada	1997 (1996)	New Brunswick, Nova Scotia, Cape Breton Island, Magaguadavic River	Atlantic salmon
Chile	2001	Region X and XI	Coho salmon
Faroe Islands [Denmark]	2000	Streymoy, Eysturoy, Kunoy, Borðoy, Suðuroy, (10 different locations)	Atlantic salmon
Ireland	2002	County Mayo, western Ireland	Rainbow trout
Norway	1984	Several counties	Atlantic salmon
United Kingdom	1998	Scotland, including Shetland Islands	Atlantic salmon
United States	2001	Maine	Atlantic salmon

## Diagnostic Manual for Aquatic Animal Diseases

In accordance with the current listed diseases in the Aquatic Code, the FDC has prepared pertinent, updated chapters in the Diagnostic Manual for Aquatic Animal Diseases. In addition to updated diagnostic chapters on the listed diseases, the Diagnostic Manual provides a general basis for health surveillance or control programs for fish, molluscs, and crustaceans. Chapters on quality management in veterinary diagnostic laboratories and principles of validation of diagnostic assays for infectious diseases are also included. These important chapters were originally prepared for the OIE Manual of Standards for Diagnostic Tests and Vaccines (for diseases of mammals, birds, and bees), but because the principles are the same for diagnostic work in aquatic animals, the FDC adapted the chapters into the Diagnostic Manual for Aquatic Animal Diseases.

The Diagnostic Manual consists of the following sections:

General provisions (definitions, quality management in veterinary diagnostic laboratories, principles of validation of diagnostic assays for infectious diseases);

Separate diagnostic chapters for diseases of fish, molluscs, and crustaceans; and a

List of reference laboratories and collaborating centers for diseases of fish, molluscs, and crustaceans.

A comprehensive approach to health control in aquatic animals requires many elements, such as:

- Assessment and maintenance of health status;
- Sampling, screening, and diagnostic methods;
- Verification of diagnoses;
- Eradication procedures; and
- Constraints of restocking in open waters and farming facilities.

Each chapter in the Diagnostic Manual is written by one or more distinguished experts in the field, based on the outline given above. These chapters describe the latest methodology for the diagnosis of the disease in question.

The Diagnostic Manual also sets standards for screening and diagnostic methods for diseases, which may be applied in any diagnostic laboratory working with diseases of aquatic animals. In addition to the more conventional methods for isolation and identification of a putative disease agent using cell cultures, culture media for bacteria and fungi, indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), bacterial isolation and identification, histology, immunohistochemistry, and standard parasitological methods, more recently developed techniques, such as the polymerase chain reaction (PCR), are described for diagnostic purposes for many of the OIE listed diseases.

The FDC is currently amending all the diagnostic chapters in the Diagnostic Manual. In the new diagnostic chapter for ISA, the FDC proposes a minimum set of criteria for suspicion and verification

of the disease. These criteria refer to reasonable grounds for suspecting fish of being infected with ISAv and the steps that competent authorities should follow to verify the presence or absence of ISA.

The proposed criteria for suspicion follow. The presence of ISA should be suspected if any of the criteria in (a) through (e) are met:

- (a) The presence of postmortem findings consistent with ISA, as described in section 2.1. of the ISA diagnostic chapter, with or without clinical signs;
- (b) Isolation and identification of ISAv in cell culture from a single sample from any fish on the farm as described in part 2.2. of the ISA diagnostic chapter;
- (c) Reasonable evidence of the presence of ISAv from laboratory tests such as IFAT (2.3.1.) and RT-PCR (2.3.2.);
- (d) The transfer of live fish into a farm where there are reasonable grounds to suspect that ISA was present at the time of the fish transfer; or
- (e) Where an investigation reveals other substantial epidemiologic links to farms with suspected or confirmed cases of ISA.

If immediate investigations do not confirm or rule out the presence of ISA, suspicion of ISA can officially be ruled out when, following continued investigations involving at least one clinical inspection per month for a period of 6 months, and no further significant evidence for the presence of ISA is obtained.

## **Confirmation of ISA**

The presence of ISA is officially confirmed if any one of these three criteria has been met.

- Clinical signs and postmortem findings of ISA in accordance with the criteria described in section 2.1.1., 2.1.2., and 2.1.3. of the ISA chapter in the Diagnostic Manual are detected and ISAv is detected by one or more of the following methods:

- (a) Isolation and identification of ISAv in cell culture from at least one sample from any fish on the farm

as described in section 2.2. of the ISA chapter in the Diagnostic Manual;

- (b) Detection of ISAv in tissues or tissue preparations by means of specific antibodies against ISAv (e.g., IFAT on kidney imprints) as described in part 2.3.1. in the ISA chapter in the Diagnostic Manual; or

- (c) Detection of ISAv by means of RT-PCR by the methods described in section 2.3.2. of the ISA chapter in the Diagnostic Manual.

- Isolation and identification of ISAv in two samples from one or more fish at the farm tested on separate occasions using the method described in section 2.2. of the ISA chapter in Diagnostic Manual.

- Isolation and identification of ISAv from at least one sample from any fish on the farm using the described method in Diagnostic Manual with corroborating evidence of ISAv in tissue preparations from any fish on the farm using either IFAT or PCR as described in the Diagnostic Manual.

If the principles given above are approved by the OIE International Committee during its general session in 2003, similar criteria will most likely be prepared in consistent manner for the other OIE-listed diseases.

## **OIE Reference Laboratories and Collaborating Center for Diseases of Fish, Molluscs, and Crustaceans**

The Diagnostic Manual also lists the OIE reference laboratories and collaborating center for aquatic animal diseases, including the name of the responsible reference expert. Currently there are 22 reference laboratories for diagnosis, control, research, and training for the OIE-listed diseases in aquatic animals as well as one collaborating center that covers more horizontal themes of importance, such as information on aquatic animal diseases. The reference laboratories share nine objectives:

- Provision of a center of expertise on a disease and standardization of methodology;
- Storage and distribution of standard strains and diagnostic standards, antisera, antigens and other reagents;
- Development of new methods;
- Collection, processing and analysis of epizootiological data;
- Provision of consultant assistance to the OIE;
- Training in specific areas;
- Organization of scientific meetings on behalf of the OIE;
- Coordination of collaborative studies; and
- Publication and dissemination of relevant information. The OIE collaborating center for information on aquatic animal diseases is located at Center for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth laboratory, U.K., and has established an international database for aquatic animal diseases. This database will also include a mapping facility to display the geographic distribution of OIE-listed aquatic animal diseases.

## **Updates of the International Aquatic Animal Health Code and Diagnostic Manual for Aquatic Animal Diseases**

Since 1995, the Aquatic Code and Diagnostic Manual has been updated regularly. It has now been decided that the Aquatic Code should be published annually while the Diagnostic Manual should be published every 2 years. Both publications may be ordered directly from the OIE in Paris. The current version of the Aquatic Code and Diagnostic Manual may also be downloaded from the OIE Fish Diseases Commission Web site ([http://www.oie.int/fdc/eng/en\\_fdc.htm](http://www.oie.int/fdc/eng/en_fdc.htm)).

## **Other Scientific and Technical Information**

The OIE Scientific and Technical Review is a thrice-yearly publication that includes comprehensive reviews, original articles, and communications. Regional conference proceedings contain the texts of communications presented at conferences of each of the five regional commissions. Other publications cover a variety of subjects ranging from brucellosis and orbiviruses to chemotherapy and successful aquatic animal disease emergency programs.

Most of these publications are available in English, French and Spanish. The Aquatic Code and Diagnostic Manual can be ordered on the FDC Web site or downloaded from the page.

## **International Conferences**

A number of international conferences on aquatic animal health matters have been organized or cosponsored by the OIE in cooperation with other organizations. The following list includes the more important ones.

Symposium on Fish Vaccination (Paris 1984)

Symposium on Problems of Chemotherapy in Aquaculture: From Theory to Reality (Paris 1991)

International Conference on Preventing the Spread of Aquatic Animal Diseases Through International Trade (Paris 1995)

OIE International Conference on Risk Analysis in Aquatic Animal Health (Paris 2000)

## **Liaison With Other International Organizations**

The OIE has formal agreements with several international organizations, such as the Food and Agriculture Organization of the United Nations, the World Health Organization, the World Trade Organization (WTO), and the Inter-American Institute for Co-operation on Agriculture. The OIE

collaborates with these organizations to ensure that close working relationships exist on debated issues concerning health controls in international trade. The OIE also enjoys privileged relations with numerous other international organizations. Table 4 gives an overview of organizations with which OIE has formal arrangements.

## **Conclusions**

Although many publications deal with the diagnosis and control of aquatic animal diseases (the American Fisheries Society's "Blue Book" [Amos 1985], the Canadian regulatory manual [Canada Department of Fisheries and Oceans 1977], European Union Council Directives 91/67 and 93/53, and the publication listed in the bibliography at Food and Agriculture Organization [2001]), the Aquatic Code, and the Diagnostic Manual so far represent the "gold standard" as the SPF Agreement of the WTO refers to the OIE as the key organization on the setting of animal health standards and guidelines.

An advantage of the Aquatic Code is that it is founded on a worldwide organization that is politically independent and that has a high level of competence in the setting of standards for the control of diseases of terrestrial as well as aquatic animals.

The Aquatic Code itself can ensure that similar standards (legal, ethical, etc.) are followed in certification. Strict ethical and moral standards are prerequisites to control and prevent the spread of diseases of aquatic animals through international trade. Appropriate certification based on sound and uniform international standards will facilitate trade and ensure that live aquatic animals and their products are free from disease(s) prevalent in the exporting country.

If the principles of the Diagnostic Manual are applied throughout the world, a uniform approach to the diagnosis of the OIE-listed diseases will be achieved. A uniform approach will help importers and exporters to meet the requirements for health certification specified in the Aquatic Code, and thus facilitate international trade.

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**Table 4—Organizations cooperating with OIE under formal agreements**

Food and Agriculture Organization of the United Nations
World Health Organization
Inter-American Institute for Cooperation on Agriculture
World Trade Organization
Organismo Internacional Regional de Sanidad Agropecuaria
Secretariat of the Pacific Community
Pan American Health Organization
World Health Organization
International Bank for Reconstruction and Development and the International Development Association (World Bank)
World Veterinary Association
CAB International on sharing of resources in the Animal Health and Production Compendium
International Federation for Animal Health
Organization of African Unity—Interafrican Bureau for Animal Resources

Council Directive of 28 January 1991 concerning the animal health conditions governing the placing on the market of aquaculture animals and products (91/67/EEC), 18 p.

Council Directive 93/53/EEC of June 1993 introducing minimum Community measures for the control of certain fish diseases, 11 p.

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# A Comparative Review of Diagnostic Assays Used To Detect Infectious Salmon Anemia Virus in the United States

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**Abstract:** Although there are several detection techniques for infectious salmon anemia virus (ISAv), none of these assays has yet been validated by reference authorities, such as the Office International des Epizooties (OIE) or the National Veterinary Services Laboratories in Ames, IA. Each diagnostic test discussed herein has problems that confound straightforward pathogen detection, interpretation of results, or both. Analytic and diagnostic variables of sensitivity and specificity for ISAv detection assays used in the United States will be discussed.

## Introduction

Detection methods for ISAv are most often used in various combinations to help veterinarians, salmon producers, and regulators decide on pathogen or disease-management strategies that directly or indirectly depend on an assay's reliability factors.

Micro Technologies, Inc., of Richmond, ME (later referred to in this paper as "the laboratory"), has refined and used several ISAv detection assays since 1998. The laboratory is approved by the U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) for the detection of 19 aquatic pathogens, including ISAv. The laboratory has collected, processed, tested, and archived many thousands of Atlantic salmon (*Salmo salar*) samples each year since 1996 for reasons of fish health certification, facilitation of movement from one facility to another, elective diagnostics, and broodstock management. The laboratory also participates in the USDA-APHIS-administered Infectious Salmon Anemia Program in Maine and provides monthly surveillance tests at active salmon production sites.

Using summaries of data collected by the laboratory since 1998, I will compare ISAv diagnostic assays for various absolute and relative correlation aspects to assess some of the sensitivity and specificity components of those assays. A good deal is at stake in establishing some of these parameters because reliable tests for ISAv detection (or for that

of any pathogen) are at the heart of risk-assessment approaches for aquatic systems. This reliability applies to the immediate needs of commercial salmon producers and agencies concerned with the status of feral Atlantic salmon and to the development of an approval rating system for farms and zones.

## Assays for Detecting ISAv

Four diagnostic assays are commonly used for detection of ISAv in Atlantic salmon and other finfish. These include cell culture, reverse transcriptase-polymerase chain reaction (RT-PCR), indirect fluorescent antibody testing (IFAT), and histopathology (Bouchard et al. 2001, Opitz et al. 2000, Lovely et al. 1999). Electron microscopy, which demonstrates the presence of viral particles, is not practical under typical diagnostic lab conditions, but it is useful as a reference standard. An enzyme-linked immunosorbent assay (ELISA) for detection of ISAv antibodies has also been developed (Kibenge et al. 2002; S. Clouthier, Maine BioTek, personal communication).

## Symptoms Associated With ISA

In addition, there is a set of physical criteria associated with, but by no means pathognomonic for, the clinical manifestation of the eponymous disease. These include exophthalmia, lethargy, darkening in external appearance, petechial hemorrhaging on the skin and surfaces of internal tissues or organs, ascites, hepatic darkening, hepato- or splenomegaly, foregut darkening, and variably pale gills and heart (Evenson et al. 1991). Hematocrits have been found as low as 10 percent or less in fish with advanced clinical disease (Thorud and Djupvik 1988). While all these conditions are nonspecific indicators of disease, in connection with mortality they may collectively allow for a tentative field diagnosis of ISA.

<sup>1</sup>Peter Merrill is an aquatic species veterinarian with Micro Technologies, Inc., in Richmond, ME.

## Lab Tests

In North America, lethal testing using primarily kidney tissue has been the norm for ISAv detection since the pathogen was first found in New Brunswick, Canada, in 1996 (Department of Fisheries and Oceans 1978, Thoesen 1994, Office International des Epizooties 2000). More recently, the laboratory has experimented with nonlethal testing techniques using blood samples for cell culture and RT-PCR. Serological tools have been developed to detect antibody (Kibenge et al. 2002), which might help assess or differentiate ISAv antibody levels in vaccinated and nonvaccinated fish. Several environmentally based assays are also in development by the laboratory to characterize epizootiological variables involved with transmission and contagion. These assays refine techniques used in RT-PCR and cell-culture testing of fish but alternatively use fomites (such as netpen materials, boat hulls, and other equipment), parasitic vectors such as sea lice (*Lepeophtheirus salmonis*), possible sentinel-type species like shellfish, sediment, and seawater itself.

There are a number of factors that potentially confound or limit correlation of results among assays. The sample choice itself is of prime importance. In Maine, Atlantic salmon are tested for ISAv for one of five principal reasons: to establish or maintain facility certification status; to transfer fish from one location to another; to screen broodstock; to monitor under the USDA-APHIS ISA surveillance program; and to electively diagnose unexplained elevated mortality. Other salmonid or nonsalmonid finfish are tested for ISAv on a surveillance basis under State or Federal programs. Objectives for these programs may be entirely different from those among commercial salmon producers. However, if ascertaining the presence or absence of the pathogen is the determinant for testing, a statistically relevant number of fish must be tested to maximize the probability of detection in a population.

## Sampling

Sample numbers for many certification programs often used a test power of 0.95 and a 5-percent presumption of pathogen prevalence (Department of Fisheries and Oceans 1978, Thoesen 1994). Thus, approximately 60 fish would be selected from any population of more than 300 individuals. However, the viral infection rate might be substantially less than 5 percent at the beginning of an epizootic, or the virus might be present in more than 5 percent of a population but not have replicated to a detectable threshold. Other factors, such as changes in viral infectivity, vaccine status, genetic strain susceptibility, nutrition, temperature, sea lice numbers, and prior therapeutic treatments, may all affect the relationship between sample selection and diagnostic information (Falk and Dannevig 1995a, Totland et al. 1996, Opitz et al. 2000). Pathogen load in the environment is probably another important variable (Nylund et al. 1994). There may be a minimal infectivity threshold for ISAv to establish itself in an individual fish or a population, but this has not been assessed per se and probably depends on many other factors which themselves would be difficult or impossible to quantify. All of these parameters are inherent but real limits to the basic sample selection process and are different from (but related to) the diagnostic sensitivity and specificity aspects of the assays themselves.

Diagnostic sample selection is often skewed to provide better diagnostic results by using moribund fish, or fish that fail to remain competitive with their cohorts (colloquially referred to as “slinks” or “pinheads”). Presumably, such fish would more likely be susceptible to ISAv infection than would apparently healthy fish. While this is probably true, it might or might not reflect actual ISAv infection dynamics. A coinfection or adverse metabolic condition might also enhance or reduce the probability of simultaneous ISAv infection. Fish for ISAv assays are commonly obtained from salmon net-pen populations during mortality collection dives, which occur with varying frequency during the production cycle. In the absence of moribund fish,

slow swimmers, or pinheads in a population, the next likely sample choice would be freshly dead fish. However, this term is subjective because the time of death is not easy to verify or visually judge.

Within the population subset used for sampling, the type and quantity of target tissues selected for ISAv detection (dependent on the particular assay) have not been standardized worldwide. The 2000 edition of the OIE Diagnostic Manual lists “spleen, heart, liver and preferably kidney tissues from *clinically infected fish*” (italics added) as the preferred sample sources for diagnostics. For cell-culture assays, the laboratory uses gill lamellae (from several hundred secondary lamellae from a 100-g fish to a dozen or so secondary lamellae from 6-kg fish), and 1-cm<sup>3</sup>-sized pieces of kidney (mid- to posterior) and spleen tissue. Tissues from no more than five fish are pooled into a single container to avoid diluting the chance of viral detection. Reproductive fluids from spawning fish, eggs, and sac-fry are also used as sample sources for ISAv tests, though there may be interference problems from cytotoxicity in the cell lines used to culture ISAv from such sources (Department of Fisheries and Oceans 1978, Thoesen 1994).

Gills are commonly collected for cell culture as part of certification screens for other pathogens of regulatory concern. Although there appears to be sufficient probability that ISAv might be detected from an infected fish with or without the use of gill lamellae (Hovland et al. 1994), additional information about ISAv presence gained from including gill tissue might outweigh the ensuing questions of whether the assay is detecting an exogenous or endogenous virion or virions. There have been several instances at the laboratory where cell culture has detected ISAv without concurring detection by simultaneous direct tissue RT-PCR. Though this situation has been rare, it might be explained if a fish were not in fact systemically infected with ISAv but carrying virus on its surface area (e.g., gills). Although the exact route of ISAv infection has not been elucidated, it may include entry through the gill lamellae (Totland et al. 1996); thus the use of gill tissue may be a worthwhile indicator of viral presence, if only in an environmental sense.

### **Tissue-Collection Techniques**

Actual collection techniques for sample tissues used in various ISAv assays may influence results. Cross-contamination of samples from different fish during collection is always possible and depends on sampler experience, transportation time constraints, fatigue, or sampling environment. Samples are sometimes collected in the field under less-than-optimal weather conditions. This may result in variability in the quantity or quality of the tissues submitted for assay. Although it is impractical to flame-sterilize equipment in the field, disinfection of collecting equipment (scalpels, forceps, etc.) is essential between samples, especially for RT-PCR assays. Utensils, or even gloved hands with residual mucus or blood, can carry enough infective tissue to cause inadvertent contamination of the assay. Minimization or avoidance of contamination can be enhanced by changing scalpel blades and gloves between cell-culture pools, after separate pen systems have been sampled, or after testing different lots of fish. Assiduous cleaning and disinfection protocols must be followed to remove extraneous organic and/or infective material between groups of samples.

The technique of collection is even more important for IFAT. Slide impressions should be made by touching the blotted surface to the slide in one or two nonsmearing motions per impression area. Excessive kidney material or bloody impressions might interfere with antibody binding. The same piece of tissue should be used for cell culture, RT-PCR and IFAT by trimming small sections for each assay. A facet of the piece of kidney tissue that is used for ISAv RT-PCR can also be used for making the IFAT slide impression, which may increase correlation between those tests.

### **Using Blood Instead of Tissue Samples**

Blood from ISAv-positive fish has the potential to be extremely useful as a nonlethal diagnostic tool, possibly supplanting the use of kidney tissue for ISAv RT-PCR. Blood smears have also been reportedly

used as adjunct ISAv assays (Office International des Epizooties 2000). Blood smears are easily made in the field and can be stained with standard Wright's–Romanowsky or other commercial stains.

### **Preserving Samples**

Sample preservation and transportation to a diagnostic laboratory are important secondary factors in the optimization of assays. Cell-culture samples are often placed into phosphate-buffered saline (PBS) during collection and sent to a laboratory for further processing. Samples should be cool (4 °C) during transport to avoid killing the virus, which ceases replication at 25 °C. (Falk 1997). These samples should be thoroughly homogenized and diluted in PBS augmented with minimal essential medium for culturing the virus within 24 h from the time of collection. Cell lines should be inoculated within 48 h thereafter. Experiences at the laboratory indicate that tissue homogenates may be frozen at –20 °C or lower for up to 3 months without substantial loss of viral recovery.

For RT–PCR, individual kidney tissues averaging around 0.25 cm<sup>3</sup> should be placed into a 1:10 (weight:volume) dilution of appropriate preservative. Samples may be left at room temperature for up to a week without loss of sensitivity but preferably should be shipped on ice and stored at 4 °C if they are not to be processed within 1 week. Samples archived for longer than 1 month should be frozen at –20 °C.

Impression slides for IFAT testing should be air-dried, fixed in acetone for 10 min, and stored in a slide box at 4 °C during shipment.

Samples for histological examination should be trimmed into cassettes and kept in a 1:10 volume:volume dilution of 10-percent neutral buffered formalin, which is changed after 24 h.

Environmental samples (e.g., seawater, sediment, mussels, swabs taken from fomites surfaces, etc.) should be placed into appropriate clean containers and kept at 4 °C during transport. Sea lice may be placed in 95-percent ethanol before

processing and shipped at 4 °C without loss of viral recovery.

Under the USDA–APHIS ISA program, attempts have been made to standardize collection, preservation, and shipping processes through uniformly training collection personnel. A USDA–APHIS-accredited veterinarian must officially sign for all samples, whether they were collected personally or through a delegate.

### **Sensitivity and Specificity**

Even a perfectly sensitive or specific assay, assuming one exists, could still be unreliable if it is performed in a way that distorts, interferes with, minimizes, artificially increases, or entirely prevents the chances of detection of the pathogen for which it was designed. A minimum of false-positive and false-negative test results, and a maximum of true-positive and true-negative test results, is the goal of all diagnostic assays. The ratios of those results, compared to some accepted standard against which all results are judged, are reflected in the sensitivity and specificity determinations of individual or combined detection assays. These determinants are reflected in positive and negative predictive values. Because none of the ISAv detection techniques have been validated, no absolute standard exists. This laboratory has modified its own protocols on many occasions to better optimize conflicting or confusing assay results. Some of these modifications are included in the review of assay techniques that follows.

### **ISAV Cell Culture**

Several cell lines are used to culture ISAv. The SHK–1 cell line (Dannevig et al. 1997), the CHSE–214 cell line (Bouchard et al. 1999), the TO cell line (Wergeland and Jakobsen 2001), and more recently the ASK cell line (see Jill Rolland's paper in this book) have been used to successfully culture ISAv. Drawbacks to cell culture include the maintenance of cell lines, the incubation timelag to initial observation

of cytopathic effects (as much as to 21 days), the interpretation of questionable cytopathic effects, and the additional steps involved in confirming cytopathic effects attributable to ISAv using RT-PCR confirmation. Nonetheless, cell culture for ISAv is generally acknowledged as the standard against which other assays are judged. The potential for false-negative results exists when using any of these three lines individually, but false positives are fewer when used in combination.

The laboratory has experienced a loss of sensitivity of the SHK-1 cell line to ISAv infection due to repeated passage. For this reason, the lab is currently evaluating the use of the ASK cell line for potential principal diagnostic use.

Cell-culture practices vary between different laboratories, and different labs use different cell-culture media and buffers (Eliassen et al. 2000, Kibenge et al. 2000, Griffiths et al. 2001, Bouchard et al. 1999). There is also a tendency to adjust the pH of the culture media according to personal biases. Time and repeated cell transfers may affect the susceptibility of a cell line to a particular virus (Wolf 1988). This laboratory therefore routinely tests the susceptibility of its SHK-1 and CHSE-214 cell lines to ISAv infection and has found that utilizing culture media at a pH of 7.2 is not only adequate for isolation of the virus but also allows for a broad range of cell-culture susceptibility to other virus isolates. Specifically for ISAv, the laboratory has demonstrated that the relatively lower pH of 7.2 has likely added to our success in culturing ISAv on the CHSE cell line (Bouchard et al. 1999). Eliassen et al. (2000) have also indicated that ISAv may require a lower pH to infect SHK-1 cells.

Cytopathic effects observed with ISAv can differ in time from inoculation to first observation, morphological changes in the cell culture monolayer, and/or the extent of cytopathic effects in either the SHK-1 or CHSE-214 lines. Cell cultures are routinely incubated for 28 d.

### **ISAv RT-PCR**

A 200-mg kidney sample should be submerged in a minimum of five volumes of RNA preservative according to manufacturers' specifications for 1 week at 25 °C, 1 month at 4 °C, or indefinitely at -20 °C without nucleic acid degradation. The tissue is considered compromised if it was not placed in RNA preservative directly after sampling from the fish and stored appropriately before and during shipment to the laboratory.

Positive controls of RNA extracted from midkidney tissue obtained from a confirmed clinical ISAv case or supernatant from an ISAv-positive cell culture are used for each run.

A commercial amplification kit is used for RT-PCR amplification. The ISAv 1D/2 primer set (Mjaaland et al. 1997, Blake et al. 1999) is used primarily at the laboratory. The FA-3/RA-3 primer set (Devold et al. 2000) may be used for confirmation of positive samples. A modified primer set has been developed at this laboratory from the ISAv 1D/2 primers for use with samples showing nonspecific background banding patterns. This phenomenon correlates with sample degradation and commonly occurs with kidney samples collected from fish that have been dead for more than 12 h. Comparison of the sensitivity of ISAv 1D/2 and FA-3/RA-3 primer sets showed no consistent differences between the two primer sets.

The RT-PCR products are typically electrophoresed on a 2-percent agarose gel at 60 v for 80 min along with a 100 base-pair DNA ladder. Gels are stained for 30 to 40 min and photographed under ultraviolet illumination. Using the ISAv 1D/2 primer set, a 493 base-pair fragment is amplified from ISAv-positive samples. Positive results are reported as an amplified band at the position where a 493 base-pair fragment would be expected to migrate, based on the location of the positive control and appropriate DNA size marker bands. The primer set FA-3/RA-3 amplifies a 211 base-pair fragment from ISAv-positive samples. Similarly, positive samples are reported as an amplified band at the

position where a 211 base-pair fragment would be expected to migrate, based on the location of the positive control and appropriate DNA size marker bands. Negative results are reported as the absence of an amplified band in the expected region. If there is any question on the size of the fragment, the sample is electrophoresed again with weak positive controls on either side of the sample for greater scrutiny.

The RT-PCR assay is prone to carryover or airborne contamination, as previously discussed. Extreme care is therefore essential in the conduct of this test.

Both PCR and RT-PCR detect the nucleic acid of an organism, in this case a negative-sense RNA virus, and therefore cannot discern between viable virus particles and nonviable particles. Theoretically, PCR can detect as little as a single genomic template. If too much RNA is used in the RT-PCR reaction, multiple banding patterns or a blur may be observed in the lane following electrophoresis, making it difficult to interpret results. Because total RNA is used in this procedure, the viral RNA is also diluted to some degree by the cellular RNA—a fact that may limit assay sensitivity. The absolute analytic sensitivity of this assay has not been determined, but in-house laboratory comparisons with cell culture indicated that RT-PCR sensitivity was an order of magnitude higher than cell culture.

The laboratory has also investigated the use of a nested ISAv RT-PCR procedure as a technique, using a second primer set (constructed of base-pair sequences contained within the first primer) to amplify products of the initial RT-PCR reaction. Comparison tests of about 100 tissue samples by both methods did not increase sensitivity.

### ***ISAv-IFAT***

Although in theory ISAv-IFAT should be both sensitive and specific (Falk and Dannevig 1995b), it is seemingly the most problematic of the commonly used assays. Sample collection and preservation processes have varied in difference to the

standardized protocol described earlier. Slides are not always collected, preprocessed, or shipped to the laboratory promptly or in the same way. Also, the steps involved in laboratory preparation of the submitted slides are numerous and technically complex and therefore become subject to cumulative artifact. Positive and negative control slides are prepared by the above technique for each batch of IFATs read at the laboratory. Positive controls are made using a 1:100 dilution of previously ISAv-inoculated cell supernatants from wells that have produced appropriate cytopathic effects. Negative controls are prepared from uninoculated cell wells.

The monoclonal or polyclonal primary and secondary antibodies may be obtained from several sources and may differ in the quantity and quality of binding and reactivity with viral antigen. Fluorescence patterns for the same slide themselves may be inconsistent when viewed with different microscopes or over time using the same microscope. Most importantly, interpretations of the gradient of fluorescence may vary with personal experience, time, number of slides viewed, fatigue, amount of ambient light, and the fluorescing wavelength of the microscope light as it changes over time. Hence, a large number of potentially confounding variables are inherent in this assay.

The gradient of IFAT scoring, from 0 (negative) to a 4+ (strongly positive), is not always a clearcut phenomenon because slides that are 99-percent “negative” (i.e., showing no detectable fluorescent reactivity) may yet have one, two, or more individual cells showing strong characteristics of positive antibody response. This can result in a “split” designation (e.g., 1+/2+, up to 3+/4+) or a qualified rating (such as “negative—two hot cells observed”). The most difficult distinction is whether to ascribe a 2+ rating or a 3+ rating to borderline cases in those categories because a 2+ rating is considered negative overall and a 3+, positive overall. The gradient of variation, as well as the absolute gradient of effect, can be continuous or discontinuous within an individual impression, between two impressions on the same slide, or between two or more slides

from the same kidney sample. Due to the poor reproducibility of fluorescent effects using black-and-white photography, visual images cannot be included in this document, but the laboratory is in the process of preparing a photographic manual of the ISAv-IFAT fluorescent spectrum for in-house and proficiency-testing use.

At the laboratory, only experienced personnel are used for IFAT reading, and at least two viewers are involved in all questionable cases before a final rating is given. With the weight of many thousands of individual ISAv assays performed over a 5-year period using different batteries of assays, it has been the lab's experience that IFAT is highly prone to false positives and false negatives alike. True positives and true negatives, however, correlate well with results by other assays. This correlation has been casually observed to occur with increasing length of time after infection. The OIE 2000 Diagnostic Manual (2000) lists IFAT as a confirmatory assay among fish exhibiting "pathological signs." Used in such a manner, IFAT results have reportedly correlated well in the field. Nonetheless, it is the consensus at this laboratory that IFAT has limited value as a confirmatory tool. While from a surveillance perspective it may be better to err conservatively in cases where farms or zones have previously tested negative for ISA, mixed diagnostic results (such as negative RT-PCR tests with accompanying positive IFATs for the same fish) can confuse salmon producers and regulators. This may cause extra labor at considerable expense for additional analysis. In areas of Canada where ISAv-positive cages or farms have been found, as few as two positive IFATs per cage have been the sole reason to depopulate production fish. This threshold has been modified recently to ensure that a total of four positive tests must be found in any cage before depopulation is undertaken. Elective action may be taken at lower thresholds.

### **Histology**

Histology is useful as a confirmatory assay after infection has caused tissue pathology. Because

"health" and "disease" are not true states of being but rather points along a continuum, there is a gradient of change in each tissue or organ system affected by ISAv infection that, taken as a whole, is representative of the syndrome. In early infection, focal congestion and dilatation of hepatic sinusoids may be evident, followed by rupture of sinusoidal endothelium and erythrocytes apparent within the space of Disse (Office International des Epizooties 2000). In later stages of disease, lesions include areas of multifocal hepatic congestion, hemorrhage, and/or necrosis that may become confluent. This process leads to a "zonal" appearance, with hepatocellular areas around large veins remaining relatively intact. In the spleen, moderate-to-severe sinusoidal congestion and erythrophagia have been reported. In kidneys, lesions are characterized by acute tubular necrosis with eosinophilic casting, and often substantial interstitial congestion and hemorrhage (Evenson et al. 1991, Falk and Dannevig 1995a, Lovely et al. 1999, Bouchard et al. 2001). Histology is not always used as a confirming tool because of the time involved in processing, the relative slowness of reading, and overall costs. Nonetheless, characteristic lesions correlate well with an assay like ISAv-IFAT.

### **Environmental Testing**

Environmental samples routinely tested for ISAv at this laboratory include seawater, cage and boat surface swabs, suspended and bottom sediment, and invertebrates (e.g., sea lice [*Lepeophtheirus salmonis*] and mussels [*Mytilus edulis*]).

Seawater is filtered through arrays of glass fiber and electronegative filters, with manipulation of the pH during various steps in order to capture any virus particles that may be present (Abbaszadegan et al. 1999, Gilgen et al. 1997). Ten L of seawater can reasonably be reduced to a 20-mL concentrate, which is used to inoculate cell cultures or is assayed by RT-PCR. The method has been successful in detecting ISAv by both assays in control samples and by one or both assays in samples not only from salmon production sites experiencing clinical ISA but

also from sites with fish testing negative for ISA-V under the surveillance monitoring program as well.

Potentially infective fomite surfaces such as harvest boat decks and hulls (Murray et al. 2001) are sampled by swabbing predetermined areas with sterile sponges. Swab samples are stored in 90-percent ethanol, concentrated through spin columns and extracted using methods similar to those used for tissues before they are assayed by RT-PCR. Swab samples to be used for inoculating cell cultures are maintained in phosphate-buffered saline and processed via routine viral culture procedures. Some ISA-V nucleic acid has been detected at the laboratory by RT-PCR from contaminated sea cages and from boat bottoms using this technique and confirmed through DNA sequencing. Parallel detection of ISA-V in swab samples by viral culture has not been observed in all samples. This may be due to the absence of viable virus particles in the presence of viral RNA.

A virus like ISA-V may have potentially multiple coinfection factors that include a variable period of incubation, an unknown *in vivo* infectivity threshold, variable and poorly characterized immunologic factors, and variable mortality; thus it might be difficult to establish what the reference standards (infection or disease) should be. Once that has been established, diagnostic assays may be further evaluated.

An ISA-V assay may at once be accurate and unbiased without being precise, sensitive yet not specific, or the converse. An assay can also be perfect in all internal and external parameters but be so expensive, time consuming, or technically difficult to perform that it cannot be employed. The laboratory assesses these parameters when developing assays.

In a practical sense, analytic sensitivity refers to the ability of an assay to detect small quantities of what it was designed to detect. Analytic specificity is similarly used to define how selective an assay is for detection of a particular pathogen. Diagnostic sensitivity and specificity are just as important to a laboratory (or researcher or regulator) from a statistical perspective. Diagnostic sensitivity is

characterized as a function of the number of positive tests it gives, in terms of true positives and false negatives. ("True" and "false" refer to whatever standard is elected against which test results are compared.) In this sense, diagnostic specificity reflects the numbers of false results an assay gives, in terms of true negatives and false positives.

### **Predictive Value of Tests**

Both the diagnostic sensitivity (D-SN) and specificity (D-SP) of an assay, or combination of assays, are integral components of the predictive value of those assays. The positive predictive value represents the probability that test subjects with positive test results actually have the pathogen or disease being assayed. The negative predictive value of an assay is the probability that test subjects with negative test results are actually free of the pathogen or disease being assayed. Predictive values of both types may then be used to establish prevalence determinations in populations. The so-called apparent prevalence can be calculated as the sum of true positives and false negatives divided by the total number of all test results. The "true" prevalence rate can be calculated as the number of true positives divided by the total number of all test results. Thus it is apparent that prevalence computations of either type, often needed to formulate or evaluate disease control programs, necessarily relate to sensitivity and specificity values for the particular assays that are used (Thrusfield 1997).

Where there are unequivocal diagnostic methods to prove or disprove test results (e.g., macroscopic pathogens such as *Myxobolus cerebralis* spores that can be visualized easily and quantified), sensitivity and specificity can be accurately computed. In the case of ISA-V and other submicroscopic organisms, sensitivity and specificity values are more easily estimated or expressed as probabilities. It is important to note again that there are many potentially confounding variables that might affect the determination of an assay's diagnostic sensitivity and specificity, such as temporal variations

in the infective process, metabolic dysfunction (a realistic concern in anadromous finfish raised in freshwater culture conditions before transfer to saltwater production locations), cross-reactivity factors for chemical components used in the test, nonspecific inhibitors or agglutinins, coinfection, toxins, immune suppression factors, and blocking antibodies.

## Comparing Tests

Calculations using data from various studies performed at the laboratory under a variety of submission types are presented to compare the sensitivity and specificity of various ISAv assays.

Data presented in table 1 below compare ISAv RT-PCR results using kidney tissue and blood with results achieved through cell culture on the SHK-1 cell line for the same samples.

Comparative D-SN and D-SP were calculated using standard formulae (Thrusfield 1997), assuming that a kidney tissue-based cell culture is the gold standard for comparison (D-SN and D-SP = 1.00). Thus, kidney-tissue-based RT-PCR results in a D-SN of 0.96 and a D-SP is 0.97. For blood-based RT-PCR, D-SN calculates as 1.00 and D-SP as 0.61.

An inference from these data suggests that using blood as a sample tissue for ISAv detection via cell culture or RT-PCR is as sensitive as using kidney tissue. At a D-SN of 1.00, blood is apparently slightly more sensitive a sample source than kidney tissue but somewhat less specific at 0.61 (compared to a specificity of 0.97 for kidney tissue). Blood,

therefore, generated more “false” PCR-positives than did kidney tissue.

Using the same fish, this time comparing IFAT procedures using kidney and blood respectively as sample sources, D-SN and D-SP for IFAT can similarly be computed. When cell culture of kidney tissue was used as the arbitrary standard for comparison of either sample source, the number of true and false positives and negatives can be calculated (table 2).

From these data, D-SN for IFAT using kidney material calculated as 0.47 and D-SP as 1.00; however, using blood as sample source D-SN was 0.04 while D-SP remained 1.00. The IFAT test using either kidney or blood smears as a sample source was much less reliable for ISAv detection because the calculated sensitivity was considerably lower than that of ISAv RT-PCR or cell culture. Interestingly, specificity for either type of IFAT was quite high in this study (1.00), as was the positive predictive value. In field use, however, this level of specificity may be offset by the low sensitivity of the assay. Because assays with low sensitivities produce high numbers of false negatives, this would not be a desirable attribute of a test designed to detect and eliminate infected animals from the population.

## Micro Technologies' Database

From January through August of 2002, the laboratory has developed a database of diagnostic information accrued from 1,053 Atlantic salmon originating among marine production sites in Maine. Under the ongoing USDA-APHIS-sponsored ISAv surveillance

**Table 1—ISAv RT-PCR sensitivity and specificity comparisons using kidney and blood from 58 fish as sample source**

Tissue, test, result	Cell culture +	Cell culture -
Kidney, RT-PCR test, +	25	1
Kidney, RT-PCR test, -	1	31
Blood, RT-PCR test, +	25	13
Blood, RT-PCR test, -	0	20

**Table 2—ISAv IFAT sensitivity and specificity comparisons using kidney and blood from 58 fish as sample sources**

Tissue, test, result	Cell culture +	Cell culture -
Kidney, IFAT test, +	8	0
Kidney, IFAT test, -	17	33
Blood, IFAT test, +	1	0
Blood, IFAT test, -	24	33

program, ISAv RT-PCR and IFAT were used to assay those samples (table 3). Cell culture was also used to retest samples based on initially positive results from either IFAT or RT-PCR. For this program, any IFAT rating of 2+ necessitated retesting even though a 2+ IFAT is ordinarily considered a negative reaction. Fish were blindly submitted for testing from sites of unknown ISAv status.

Eight positive ISAv RT-PCR results and 171 non-zero-graded IFATs were obtained; some results were from retesting of sites with positive tests. Fluorescent antibody results produced 111 samples with a 1+ rating, 45 with 2+ rating, and 15 with a 3+ rating. No 4+ IFAT results were observed. Each of the 8 ISAv-positive RT-PCR results, and 11 of the 15 3+ IFAT ratings came from a site with subsequently confirmed ISAv infection. Excluding that site, the remaining 160 non-zero-graded IFATs were not supported by RT-PCR results. Such disagreement may reflect variables associated with sensitivity or specificity of the assays, viability of the pathogens, or other unknown factors.

**Table 3—Comparative surveillance testing results for ISAv from 1,053 Atlantic salmon**

RT-PCR results		ISAv IFAT results			
Neg.	Pos.	0	1+	2+	3+
1,045	8	882	111	45	15

## Results From a Two-Lab Study

In late 2001, a study examining several comparative diagnostic parameters was undertaken between 2 labs using a total of 60 Atlantic salmon exposed either naturally in the field (and logically through subsequent cohabitation in the lab tanks) or experimentally exposed to ISAv, along with 2 negative controls. Assays included ISAv RT-PCR using blood and kidney tissue in addition to virus isolation using SHK-1 and CHSE-214 cell lines on individual fish pools. Hanks' balanced salt solution was used as a transport medium for this study as a comparative sample preservative.

Although 62 percent (37 of 60) of fish selected for inclusion in this study from the field had relevant clinical signs, fish not demonstrating clinical signs also tested ISAv-positive by various assays. Inspection of the PCR testing results indicated that 100 percent of the fish were infected with or carrying ISAv (table 4). There was good interlaboratory correlation for total ISAv RT-PCR results using either blood or kidney tissue. Excellent correlation also existed between blood and kidney as sample tissue for the ISAv RT-PCR assay. One immediately apparent difference in results is for virus isolation using SHK-1 cells, where one lab failed to culture any virus from more than 60 samples that had tested overwhelmingly positive through ISAv RT-PCR. The other laboratory cultured ISAv from the population with both cell lines, although at the success rates of

**Table 4—Comparative diagnostic parameter assessments for ISAv tests, 2001**

Fish origin	# of fish	Clinical signs+	RT-PCR (kidney)		RT-PCR (blood)		SHK VI+ kidney/blood		CHSE VI+ kidney/blood*	
			Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2
Neg. control	2	0	0	0	0	0	0/0	0/0	N/a	0/0
Naturally exposed	20	18	19	20	20	20	0/0	12/7	N/a	4/1
Experimentally infected	40	19	40	40	40	37	0/0	18/18	N/a	9/12
Totals	62	37	59	60	60	57	0/0	30/25	N/a	13/13

\*Lab 1 did not perform culture using CHSE-214 cells.

50 percent v. 42 percent for SHK-1 cells using kidney and blood, respectively, as sources for viral isolation. Only one laboratory used the CHSE-214 line, with a culture rate of 22 percent for both kidney and blood. From these data, blood appears to be somewhat less sensitive a tissue choice for cell culture than kidney, resulting in a 17 percent lower culture rate with SHK-1 cell culture. The CHSE-214 line overall had a 56 percent lower successful culture rate compared to SHK cells. Based on this apparent difference, the SHK-1 cell line was more sensitive to ISAv infection than the CHSE-214 line, although historically the laboratory has consistently cultured ISAv successfully using both lines. The use of both cell lines for concurrent cell culture assays for ISAv is recommended to increase the overall sensitivity of the test. Both labs produced similar results on negative control samples.

## **IFAT Produces Variable Results**

Other interlab exercises have been performed at periodic intervals, and the usual variant among laboratories was results produced for IFAT ratings. This is particularly true at the lower end of the gradient, where labs may disagree on what specifically constitutes a 1+ or 2+ rating. While still in the negative category, there is a substantial qualitative and quantitative difference between the extremes of the negative range. In part, as mentioned in earlier sections, there may be differences in collection, preservation, preparation, or interpretation. One other possible factor affecting interpretation is the number of fields read per slide. While in theory the entire slide is scanned during the evaluation process, in practice fewer than the potential total number of fields may actually be read, depending on the size of the impression, number of slides to read, time constraints, etc. This phenomenon has been noted many times at our laboratory, and a logical conclusion that may be drawn is that the overall negativity of a slide may be a function of the time taken for reading and the number of fields viewed.

A true ISAv assay validation study for any assay using the OIE-recommended number of 2,000 test animals has yet to be published but will be a necessary component of rational ISAv/ISA management approaches internationally. Although RT-PCR appears to be at least as diagnostically sensitive as cell culture for viral detection, the significance of the results from RT-PCR is debated. There is apparently enough variation between available cell lines used for virus isolation that each should be validated in its own right. Fluorescent antibody tests appear to be useful as a detection tool at later stages of infection, but that is mainly an anecdotal conclusion without supporting, published evidence. Experiences over the past 3 years at this laboratory have demonstrated that IFAT results alternately correlate and disagree not only with results from other assays but within a single diagnostic submission as well.

## **Standards Are Needed**

Reference institutions and resource agencies need to provide the standardizing framework for both the available and developing ISAV detection assays. The determination of a gold standard with acceptable levels of sensitivity, both generally and for particular ISAv assays, must be defined. This determination depends upon the nature of the testing program being utilized. If the goal of the program is to detect and eliminate ISAv-infected fish, a highly sensitive and fairly specific test is needed. Such an assay would have relatively few false negatives but produce some false positives. Alternatively, if the goal is to confirm the results of another assay, a very specific test with reasonable sensitivity would suffice to avoid false positives. The degree of acceptable levels of both sensitivity and specificity will play a deciding role in these respects. Sample size also is a determinant in the reliability aspect of the diagnostic equation because, at the group or population level, sensitivity and specificity are influenced by sample size. With low prevalence levels of a pathogen, as may be the case with initial ISAv infection in a population, even

very reliable tests with high sensitivity and specificity have a relatively low predictive value. Because prevalence can change over time, the anticipated predictive value of an assay should be periodically reviewed in context to the situation as it becomes better characterized statistically.

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# The Development of Infectious Salmon Anemia Virus Vaccines in Canada

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**Abstract:** Infectious salmon anemia virus (ISAv) is currently one of the most important viral pathogens threatening commercial aquaculture in the Northern Hemisphere. Morphological, biochemical, and replication properties, which are similar to those of the influenza viruses, indicate that ISAv is a member of the *Orthomyxoviridae*. Comparison of ISAv proteins with other orthomyxoviruses revealed low amino acid identity values (between <13 percent and <25 percent), supporting the proposal to assign ISAv to a new genus, *Isavirus*, within the *Orthomyxoviridae*. Infectious salmon anemia in the Bay of Fundy, New Brunswick, is now a managed disease following the compensation scheme agreed to by the Canadian Federal Government in 1999, and the various steps taken by the industry. These steps include the adoption of stringent husbandry practices, an ISA surveillance program, depopulation of affected sites, and vaccina-

tion. However, the ISAv vaccines currently used are not 100-percent protective. Immunized fish do not clear the virus, and they may become carriers, which makes control by vaccination incompatible with depopulation control methods. There is still an absolute need to match the ISAv vaccine composition to current viruses and to improve on the immunogenicity of ISAv vaccines. Studies on more than 160 ISAv isolates have established the existence of two hemagglutinin (HA) subtypes of ISAv, one North American and one European, and four distinct neuraminidase (NA) subtypes, indicating the existence of up to eight different combinations of HA and NA subtypes among isolates. These observations are discussed in relation to new vaccine developments for ISA. An enzyme-linked immunosorbent assay (ELISA) that detects fish antibodies to ISAv was also developed and can be used to assess vaccine efficacy.

## Introduction

Infectious salmon anemia (ISA) is an emerging, highly fatal viral disease of marine-farmed Atlantic salmon (*Salmo salar*) that is negatively affecting the salmon-farming industry in an increasing number of countries, particularly in the Northern Hemisphere. The ISA virus (ISAv) has caused disease in Norway since 1984 (Jarp and Karlsen 1997), and the disease was probably present in Norwegian salmon farms as early as 1977 or 1978 (Devold et al. 2001). Although ISA outbreaks in New Brunswick, Canada, have been recognized since 1996 (Bryne et al. 1998, Mullins et al. 1998, Lovely et al. 1999), there is anecdotal evidence that the virus was present in the Bay of Fundy by 1995. ISA outbreaks have occurred in the Faroe Islands, Denmark (Anonymous 2000), and in Maine, U.S.A., since 2000 (Bouchard et al. 2001). Clinical disease also occurred in Scotland in 1998 and 1999 (Rodger et al. 1998, Murray this volume) and in Nova Scotia in 2000 (Ritchie et al. 2001). In August 2002, ISAv was also isolated for the first time in Ireland, from fish on a rainbow trout farm in Clew

Bay, County Mayo, without evidence of a clinical outbreak (Anonymous 2002). Although there is still controversy about the occurrence of ISAv in Chile [Pedro Smith informed us in New Orleans that the nonstandard illness in Chilean coho salmon was not actually ISA despite appearances], laboratory data on tissues and sera from farmed coho salmon (*Oncorhynchus kisutch*) support its existence in that country as well (Kibenge et al. 2001a, 2002).

Clinical disease in farmed Atlantic salmon is characterized by high mortality, exophthalmia, severe anemia, ascites, hemorrhagic liver necrosis, and renal interstitial hemorrhage and tubular necrosis. Currently, each country has its own procedures in place to deal with ISA outbreaks. The most dangerous infections are the subclinical ones that maintain the virus within known geographic areas. It is now suggested that the disease is caused by novel virulent strains of ISAv that may emerge from background benign infections in the wild fishery and then adapt to intensive aquaculture practices (Murray et al. 2002). The virus appears to be well established in the wild fishery (Raynard et al. 2001) and cannot therefore be eradicated. Thus, the aquaculture industry will have to learn to manage it. In New Brunswick, ISA is now a managed disease following the compensation scheme agreed to by the Canadian Federal Government in 1999 and the various steps taken by the industry, including stringent husbandry practices and vaccination.

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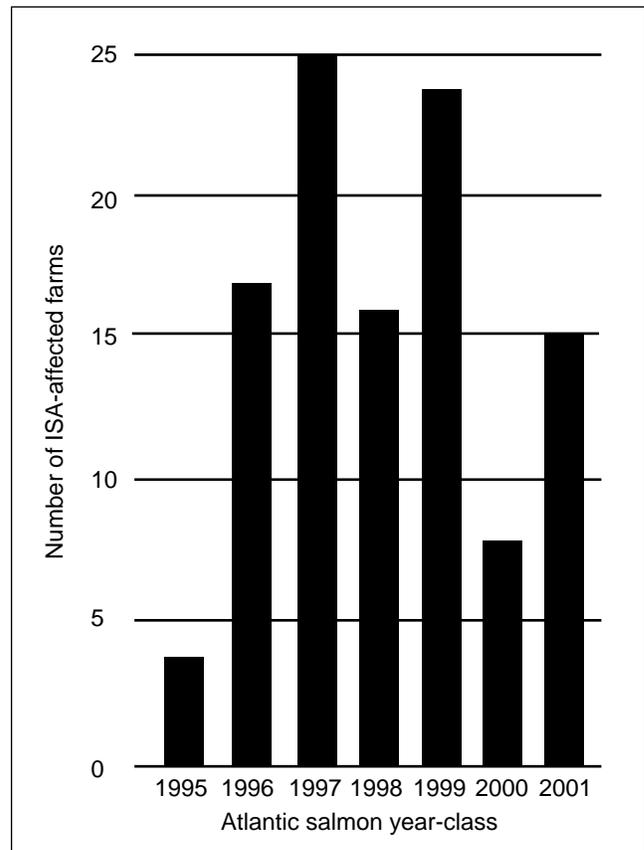
The properties of ISAv indicate strongly that the virus belongs to the family *Orthomyxoviridae* and is similar to influenza viruses. Given the high mutability of these viruses in their surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA), which are the most important targets for the host immune system, it is absolutely essential that the ISAv vaccine composition be matched to the actual circulating viruses. In humans, new strains of influenza A virus arise every 1–2 years as a result of selected point mutations in these two proteins—a phenomenon known as antigenic drift. Sometimes, an exchange of the HA or NA gene segment, or both, with a strain of influenza A virus of another subtype occurs (a phenomenon known as antigenic shift), and this may result in an influenza pandemic. Consequently, human influenza vaccines are updated yearly to induce immune responses specific for the prevalent strains. These vaccines are efficacious when the HA of the vaccine matches that of the circulating virus but not when it differs as a result of antigenic drift or shift.

This paper discusses the current ISA vaccine effort in Canada and highlights important challenges related to ISAv strain variation, immunogenicity, and public education. These concepts are important to understand (1) why disease control by vaccination is incompatible with current depopulation control methods, (2) what new vaccine developments have been achieved, and (3) what indicators of ISAv vaccination are desired for vaccines to have substantial preventative value.

## Incidence of ISA in New Brunswick, Canada

Initial diagnosis of ISA in the Bay of Fundy was delayed because of the preponderance of lesions in the kidney (rather than the liver) of affected fish, and the disease was initially termed hemorrhagic kidney syndrome (HKS) by Byrne et al. (1998). Laboratory confirmation of ISAv in HKS was also initially delayed because of the presence of a novel Toga-like virus (that was cytopathic in CHSE–214 cells) in mixed

infection with ISAv (Kibenge et al. 2000a). It was not until Canadian laboratories had access to the SHK–1 cell line that confirmation of the etiological role of ISAv in HKS became possible (Lovely et al. 1999). Moreover, the New Brunswick ISAv isolates showed phenotypic variation in that some isolates were cytopathic on CHSE–214 cells (Bouchard et al. 1999), whereas others were not (Kibenge et al. 2000b). Until August 1997, only the premarket classes of fish (> 1 kg) were identified with HKS. Since August 1997, mortality associated with the syndrome has been reported in the smolt year-class (about 300–400 g). As of August 2002, ISA outbreaks in the Bay of Fundy (fig. 1) have cost the industry in excess of \$50 million Canadian.



**Figure 1**—Estimated number of farms affected with ISA in New Brunswick by August 2002. [Data provided by Dr. Sandi McGeachy, New Brunswick Department of Agriculture, Fisheries and Aquaculture.]

## Current ISAv Vaccine Usage in New Brunswick

Increased survival of fresh-water-reared Atlantic salmon vaccinated with inactivated whole ISAv emulsified with mineral oil and then experimentally infected with ISAv has been reported (Brown et al. 2000, Jones et al. 1999b). This is the basis for the current ISAv vaccines used in Canada. However, it had been recognized long before this that the immune response in Atlantic salmon did not provide full protection against the disease, for mortality could be produced both in fish that had previously recovered from experimental ISAv infection and those that were passively immunized with serum from fish that had recovered from ISA (Falk and Dannevig 1995). The first commercially available ISAv vaccine was an autogenous product using the ISAv isolate NBISA01 (Jones et al. 1999a,b). All 1999 fish stocked in the New Brunswick parts of the Bay of Fundy were vaccinated with this vaccine. This vaccine has since been licensed in Canada and the

United States as Forte V1 by Novartis (Aqua Health). Since 2001, a second inactivated ISAv vaccine (Brown et al. 2000) has also been marketed in New Brunswick. For all intents and purposes, practically all farmed Atlantic salmon currently stocked in the Bay of Fundy are vaccinated against ISA.

Several potential outcomes might result from vaccination against ISAv (table 1). Vaccination will only have substantial preventive value if the ISAv vaccines induce broadly neutralizing antibodies and the immunized fish do not become virus carriers. The 1999 autogenous vaccine was not completely effective because more than 1 million of the vaccinated fish had to be removed early after some of them tested positive for ISAv. In addition, fish at several new sites became infected. However, ISA outbreaks have continued to occur even when the licensed ISAv vaccines, which have controlled antigen content and long-term stability, have been used (McGeachy 2001). A major challenge with current ISAv vaccine use in Canada is that the field performance of these vaccines cannot be adequately evaluated because of the control strategy whereby any cage with in excess of 0.05 percent mortality per week is depopulated. Surveys for ISAv in Atlantic salmon by reverse-transcriptase–polymerase chain reaction (RT–PCR) on different fish farms have disclosed that virus was present in fish without clinical signs of disease from some cages (McClure and Hammell, this volume). This fact suggests that, although early harvest of cages involved with outbreaks removes a source of the virus, the unaffected cages that remain on a positive farm may become a reservoir of the virus. However, these nonclinical cages may be the ones in which the ISAv vaccine is protective against clinical disease (table 1, outcome 3). Thus, the immunity engendered by these vaccines is neither 100 percent protective nor sterile.

Researchers in a recent laboratory study (McDougall et al. 2001) observed that vaccine efficacy correlated with protection from mortality (clinical disease) and elimination of virus in challenged fish, but surviving fish tested positive for the virus.

**Table 1—Potential outcomes and indicators of vaccination against infectious salmon anemia virus (ISAv)**

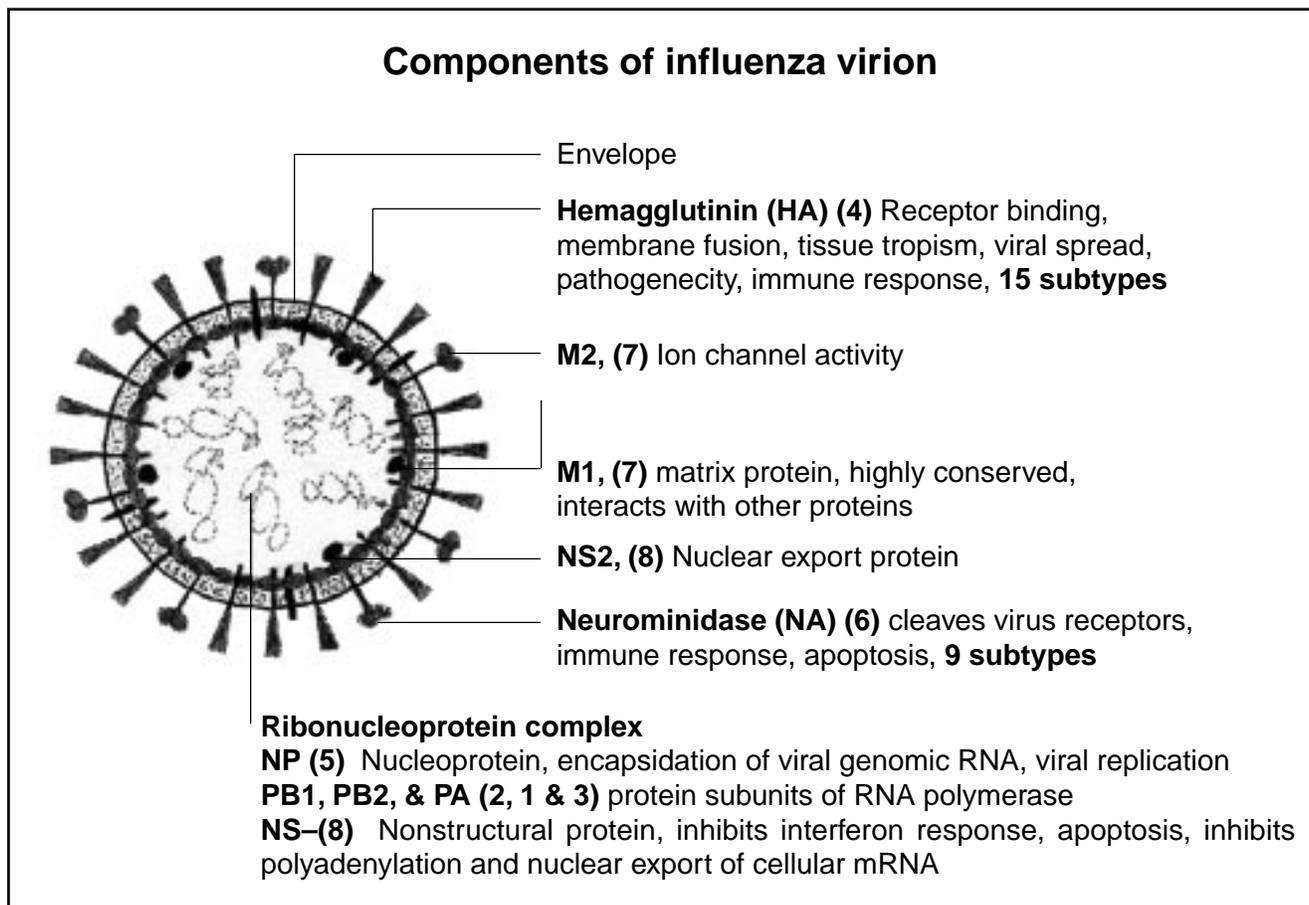
Outcome	Indicators
1. Sterile immunity	<ul style="list-style-type: none"> <li>• No virus detected using the most sensitive RT–PCR assay</li> <li>• No antibodies to ISAv nonstructural proteins<sup>1</sup></li> <li>• No clinical disease</li> </ul>
2. Transient subclinical infection	<ul style="list-style-type: none"> <li>• No virus transmission to naïve fish</li> <li>• RT–PCR positive</li> <li>• Antibodies to ISAv nonstructural proteins present</li> <li>• No clinical disease</li> </ul>
3. Controlled subclinical infection	<ul style="list-style-type: none"> <li>• Virus isolation positive (virus carrier)</li> <li>• Antibodies to ISAv nonstructural proteins present</li> <li>• No or reduced mortality (mild clinical disease)</li> </ul>
4. Enhanced clinical infection	<ul style="list-style-type: none"> <li>• Very high mortality (severe clinical disease)</li> </ul>

<sup>1</sup> If salmon were vaccinated with inactivated whole ISAv.

## The Concept of Strain Variation in ISAv

The morphological, biochemical, and replication properties of ISAv indicate strongly that this virus is a member of the *Orthomyxoviridae* family (Kim and Leong 1999), and it is similar to influenza viruses (fig. 2). The single-stranded RNA genome of ISAv comprises eight segments of negative polarity, ranging in size from 1.0 to 2.4 kb with a total molecular size of approximately 14.3 kb (Clouthier et al. 2002). Comparison of the ISAv proteins with those of other orthomyxoviruses revealed low amino

acid identity values that were between <13 and <25 percent (Kibenge et al. 2001b, Krossøy et al. 1999, Ritchie et al. 2002, Snow and Cunningham 2001). These results support the proposal to assign ISAv to a new fifth genus, *Isavirus*, within the *Orthomyxoviridae* family (Anonymous 2001). The concept of ISAv strain variation has gained acceptance among researchers since Blake et al. (1999) first reported genomic sequence data showing significant differences between Canadian and Norwegian isolates. Our laboratory has studied isolates from different geographic regions and provided the first direct proof that such isolates also show phenotypic

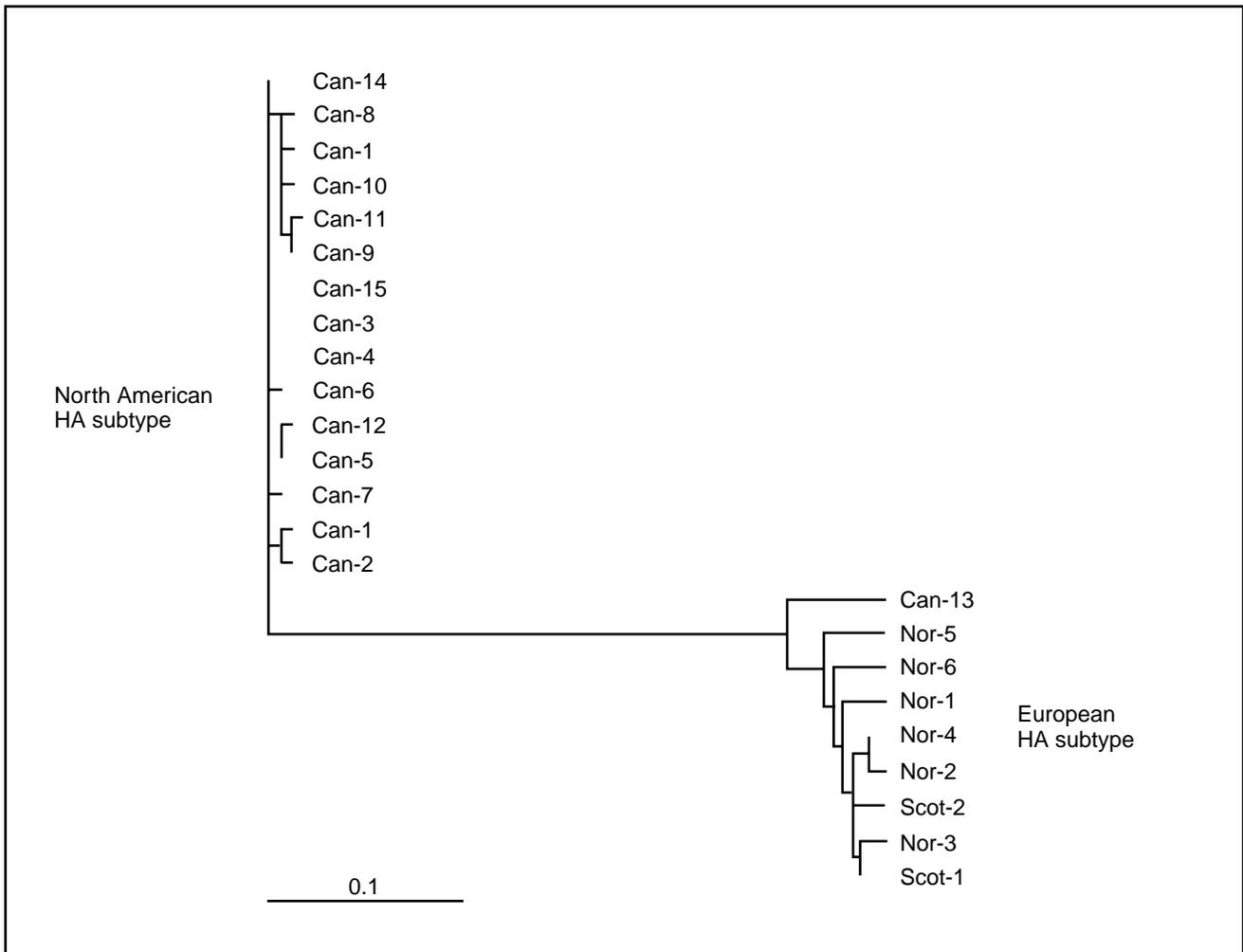


**Figure 2**—Components of the influenza virion. The numbers in brackets refer to RNA genomic segments encoding the corresponding viral proteins in influenza A virus.

variation (Kibenge et al. 2000b, 2001b). It is possible that the virus is mutating in the wild fishery, and the strain variation that was detected (Blake et al. 1999, Devold et al. 2001, Kibenge et al. 2000b, 2001b) may be a reflection of the wide range of natural marine fish hosts.

A previous study of 32 ISAv isolates (Kibenge et al. 2001b) identified two HA subtypes, one North American and one European, on the basis of neutralization with rabbit antisera to whole virus and by sequence analysis of the HA gene (fig. 3).

Recently, we compared the NA gene sequence (ISAv RNA segment 5) among 30 ISAv isolates within the 2 HA subtypes. Our results revealed up to four distinct NA subtypes, indicating the existence of eight different combinations of HA and NA subtypes among ISAv isolates (table 2). Six of the HA/NA subtypes were identified among North American isolates, and four were identified among European isolates. Each geographic region had one predominant HA/NA subtype. These data have significant implications with regard to ISAv vaccines



**Figure 3**—Phylogenetic tree showing the relationship between different ISAv isolates: the two HA subtypes, one North American and one European, are evident. For a more detailed analysis, refer to Kibenge et al. (2001b).

**Table 2—Hemagglutinin (HA) and neuraminidase (NA) subtypes of ISAv from North American and European sources**

HA and NA Type <sup>1</sup>	H1N1	H1N2	H1N3	H1N4	H2N1	H2N2	H2N3	H2N4
Number of ISAv isolates	11	2	3	2	2	5	1	4

<sup>1</sup> H1 corresponds to the North American HA subtype; H2 corresponds to the European HA subtype; N1–N4 correspond to the NA (RNA segment 5) genotypes.

used in the Bay of Fundy because they show (1) that virus-neutralizing antibodies to ISAv are subtype specific, (2) that both HA subtypes are present in New Brunswick, and (3) that there may be more antigenic variation if the NA variation is considered. Thus, candidate vaccines that induce broadly neutralizing antibodies are needed to provide improved protection against ISAv infection, clinical disease, or both.

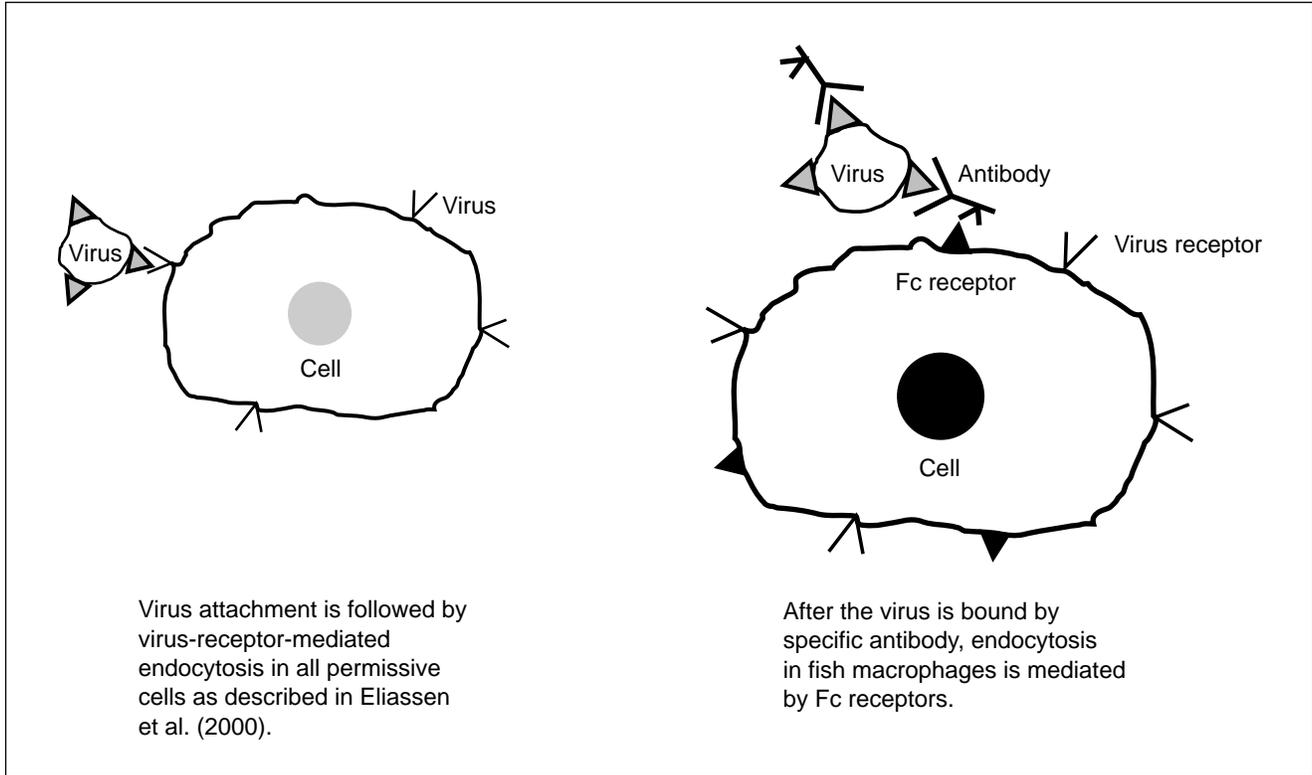
## Immunogenicity of ISAv

Most commercially available viral vaccines for fish contain inactivated whole virus as antigen. Developments in protein expression technology and vaccinology have made it possible to combine safety benefits of subunit (SU) vaccines with the advantages of live vaccines. Such benefits include the use of lower antigen mass, elicitation of long-lasting immune memory, and stimulation of mucosal immune responses as well as effective cell-mediated immune responses by using naked DNA vaccines. These new approaches to disease prevention in aquaculture represent a radical change in the way that antigens are delivered. DNA immunization involves the direct introduction of plasmid DNA encoding an antigenic protein, which is then expressed within the cells of the host animal. This expression leads to the development of surprisingly strong immune responses involving both humoral and cellular immunity.

Successful DNA vaccinations of fish against infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) have already been reported (Boudinot et al. 1998, Corbeil

et al. 2000). The nucleotide sequence information of all the RNA segments of ISAv and the putative proteins that are encoded have been published (Clouthier et al. 2002). These developments have opened the way to production of SU and DNA vaccines against ISAv. Recombinant monomeric HA candidate vaccines are bound to elicit neutralizing antibodies that are subtype specific. Thus, to induce antibodies with broad activity, either mixtures of recombinant proteins or highly conserved domains of viral proteins may be necessary. In mice, vaccines inducing antibodies to the highly conserved extracellular domain of the M2 protein conferred protection to influenza A virus infection (Neiryneck et al. 1999). DNA vaccines for influenza virus showed that coimmunization with influenza HA + NP DNAs enhanced protective immunity (Pertmer et al. 2000). Immunization of mice with a conventional inactivated trivalent influenza vaccine supplemented by two NA subunits of different subtypes was efficacious in protecting against homotypic and heterotypic influenza viral challenges (Johansson et al. 2002). However, vaccination of pigs with a DNA construct expressing an influenza virus M2–nucleoprotein fusion protein exacerbated clinical signs of disease after challenge with influenza A virus (Heinen et al. 2002).

Recently, we have discovered a unique phenomenon of ISAv infection in the macrophage-like cell lines SHK–1 and TO that suggests that antibodies to some ISAv antigens may be harmful because they facilitate internalization of antibody-bound virus and growth in the cells (fig. 4). This phenomenon was not seen when we used the TO cells with infectious pancreatic necrosis virus (IPNV)



**Figure 4**—Illustration of proposed mechanisms by which ISAv may infect fish macrophages.

**Table 3—Virus neutralization against ISAv and infectious pancreatic necrosis virus (IPNV) in different fish cell lines**

Cell line	Virus (strain)	Rabbit anti-ISAv serum titer <sup>1</sup>	Rabbit anti-IPNV serum titer <sup>1</sup>
CHSE-214	ISAv (NBISA01) IPNV (FVX73)	640 (640) ND	ND 10,260
SHK-1	ISAv (NBISA01) IPNV (FVX73)	20 ND	ND ND
TO	ISAv (NBISA01) IPNV (FVX73)	20 (960) ND	ND 10,260

<sup>1</sup> Virus neutralizing (VN) antibody titer expressed as the reciprocal of the highest dilution of serum to completely neutralize 100 TCID<sub>50</sub> of virus. The number in brackets is the VN antibody titer in the presence of staphylococcal Protein A; ND denotes not done.

and rabbit antiserum to IPNV (table 3). Thus, harmful ISAv antigens would need to be identified and excluded from ISAv vaccines in order not only to avoid exacerbating clinical disease (table 1, outcome 4) but also to achieve sterile immunity in fish (table 1, outcome 1).

Current vaccine delivery by injection is also not suitable for dosing large numbers of fish in a very cost-effective manner. There is, therefore, a need to develop live recombinant vectors for gene delivery to fish organs such as the gills, the skin or the lateral line, and the gut. Such organs are believed to be important in the antigen uptake and in the induction of immunity during vaccination by immersion (Tatner et al. 1984). However, the potential for environmental contamination dictates that efforts focus on development of recombinant viral vectors with very limited or no ability to replicate in fish cells.

One significant advance in the field of ISAv vaccine development would be determination of the immune correlates—the type, magnitude, breadth, and/or location of immune responses—associated with protection against infection and clinical disease. Atlantic salmon antibody response to ISAv studied by Western blotting revealed that the antibodies bound exclusively to the viral nucleoprotein (Falk and Dale 1998). Although previous studies reported increased resistance of Atlantic salmon to ISAv upon reinfection or after passive immunization with serum from fish that had recovered from ISA (Falk and Dannevig 1995), or following vaccination with inactivated virus (Brown et al. 2000, Jones et al. 1999b), antibody levels in such fish were not determined. To date there has been no report on laboratory assessments of ISAv-vaccine-induced immune responses

Fish serology could be important for assessing ISAv vaccine efficacy. Our research has established a protocol for detecting fish antibody to ISAv using ELISA with purified whole virus as the coating antigen and monoclonal antibodies that detect fish immunoglobulins bound to the antigen on the ELISA plate. According to this ELISA system, farmed fish presented two different types of antibody responses to ISAv. Naturally infected Atlantic salmon carrying ISAv that was detected by RT-PCR had a specific antibody response to ISAv suggestive of recent infection. Those fish that were virus negative by RT-PCR had an elevated, nonspecific antibody reactivity possibly suggestive of chronic infection or resistance to ISAv (table 4). Sera from experimental fish collected up to 6 weeks after infection with ISAv do not show the elevated, nonspecific antibody reactivity.

## Public Perception of New Vaccine Developments

The fish vaccine industry is developing live vaccines, DNA vaccines, and SU vaccines as alternative approaches to vaccinate against fish viral disease. The industry is also pushing for greater acceptance of these methods of vaccine delivery. However, problems arise from various regulatory authorities

**Table 4—Infectious salmon anemia virus RT-PCR and antibody ELISA results in Atlantic salmon (Kibenge et al. 2002)**

Fish number <sup>1</sup>	RT-PCR <sup>2</sup>	ELISA OD405 on ISAv antigens	ELISA OD405 on SHK-1 cellular antigens
F01	–	<sup>3</sup> 0.35 ± 0.027 (–)	0.39 ± 0.028
F02	–	0.234 ± 0.001 (–)	0.54 ± 0.03
F03	+	0.3 ± 0.021 (+)	0.025 ± 0.004
F04	+	2.14 ± 0.3 (+)	0.0
F05	–	0.28 ± 0.045 (–)	0.11 ± 0.001
EF06	+	0.31 ± 0.016 (+)	0.061 ± 0.038
EF07	–	0.009 ± 0.001 (–)	0.02 ± 0.002

<sup>1</sup> F01–F05 were samples from farmed Atlantic salmon; EF06 and EF07 were positive and negative control experimental fish, respectively.

<sup>2</sup> RT-PCR-assayed kidney tissues.

<sup>3</sup> Parentheses denote ELISA negative (–) and positive (+) results.

with all three of these approaches. In addition, the public, the regulatory authorities, and politicians must be educated concerning such deliveries. For example, in several countries, a DNA-vaccinated fish is classified as a genetically modified organism (GMO) even though there is no evidence that it is genetically modified. Public perceptions do not favor production of a GMO salmon. If the public will not eat the salmon, the fish farmers cannot use this vaccine. Thus, there is an absolute need to educate the public, the regulatory authorities, and the politicians concerning new vaccine modalities.

## Conclusion

ISA is now a managed disease in Canada. Practically all fish stocked in the Bay of Fundy are vaccinated with inactivated whole ISAv antigens. Clinical disease occurs, albeit at lower levels than in 1999, and virus is present even in apparently healthy fish. Thus, the immunity engendered by the vaccines is neither 100 percent protective nor sterile. Because of the possible multiplicity of the ISAv HA/NA subtypes in existence, vaccines that induce broadly neutralizing antibodies are needed to provide improved protection against infection and clinical

disease. Harmful antigens need to be identified and excluded from ISAV vaccines to achieve sterile immunity in fish. The public, the regulatory authorities, and politicians need to be educated about the new vaccine developments such as live (recombinant viral vector) vaccines, DNA vaccines, and SU vaccines. One achievement that would significantly advance the field of ISAV vaccine development is the elucidation of immune correlates that are associated with protection against infection and clinical disease. A newly developed antibody ELISA shows two different types of antibody responses to ISAV by farmed fish: a specific antibody response in virus-positive fish and a nonspecific antibody reactivity in virus-negative fish that may be resistant to ISAV.

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# Epidemiologic Study of Infectious Salmon Anemia in Maine: Combining the Subjective With the Objective in Risk Factor Estimation

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**Abstract:** Infectious salmon anemia (ISA) was first officially documented in Maine in February 2001. The disease crippled Maine's salmon aquaculture industry, leading to the mandatory depopulation of 1.6 million fish in the winter of 2001–02. We provide a preliminary report of our efforts to track the spread of the virus and identify risk factors associated with this epidemic. We focus on several points of discussion, including:

(1) industry's hesitancy to share production records owing to concerns over public access of the data;

(2) our attempts to employ subjective probability estimation techniques to circumvent these confidentiality issues; (3) our plans to follow this subjective process with a targeted analysis of coded empirical data; and (4) the additional benefit of opinion-based assessments in their relative ease at bridging political boundaries.

This last point is of great importance to the ISA program because Maine and New Brunswick waters are primarily separated by management and political, rather than spatial, dimensions.

## Introduction

The U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) currently oversees surveillance for infectious salmon anemia virus (ISAv) in farmed salmon operations throughout eastern Maine. As a part of this program, USDA has requested the collection and analysis of surveillance data for epidemiologic assessment. Consequently, we are assimilating and organizing data to identify key risk factors that may help prevent or mitigate future ISA outbreaks. However, recent litigation and allegations that the salmon industry is currently experiencing have caused great concern over the possible repercussions of the further sharing of proprietary data. In this paper, we outline some of the steps that we are taking to ensure accurate and complete reporting without causing the industry further vulnerability.

## The Problem

Epidemiologic studies of ISA in Norway; New Brunswick, Canada; and Scotland (Vagsholm et al. 1994, Jarp and Karlsen 1997, Hammell and Dohoo 1999, Stagg et al. 2001) target certain management practices such as shared equipment, multigenerational farms, farmed fish movements, and stocking density as potential intervention points for disease

control. Experience gained from the history of disease in these countries has helped guide efforts to control ISA in Maine. However, many questions regarding disease transmission and risk remain unanswered.

ISA reports in Cobscook and Passamaquoddy bays do not track a clear pattern but rather move from site to site and even pen to pen in seemingly random fashion. Many salmon farms in Maine kept detailed production records throughout the epidemic, and anecdotal accounts of the months preceding salmon depopulation in Maine are packed with extremely interesting and potentially very useful information. Epidemiologic evaluation of the ISA outbreak in Maine's Cobscook and Passamaquoddy bays could help further define how ISAv disseminates; why certain pens, sites, and fish are more vulnerable to infection than others; how to predict farms at greatest risk; and how to mitigate consequences of future outbreaks.

We had initially hoped to survey farms in eastern Maine on management practices, site locations, environmental characteristics, and ISA disease incidences and impacts. However, we have found that, despite the inherent value of the data generated during the epidemic, answers to our questions are extremely difficult to obtain. Initial inquiries into the willingness of companies and veterinarians to participate in an epidemiologic analysis of retrospective data on ISA met with varied levels of enthusiasm. Most support the effort but are very hesitant to offer direct access to empirical data.

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The reasons for this guarded response are clear. Authors Gustafson and Ellis represent a Federal agency, and therefore this work is technically open to public scrutiny in the United States through the Freedom of Information Act. Clearly, much of the information that we are requesting is proprietary. This is of particular concern to the industry in light of the recent litigations against salmon companies in Maine: one in which salmon farms were held in violation of the Clean Water Act followed soon thereafter by another in which a farm was found guilty of delaying ISA reports to the Department of Marine Resources. The acquisition and inappropriate use of epidemiologic information by interest groups lacking expertise in aquatic animal health could seriously damage legitimate disease-control efforts. Consequently, we have had to change our focus and approach, though not necessarily our goals.

## **Potential Solutions**

We see two adjoining avenues for obtaining sensitive information without threatening company security or trust: (1) develop a subjective database in the absence of empirical data, and (2) code a limited set of empirical data to ensure confidentiality.

### **Opinion-Based Data**

We are employing subjective probability estimation techniques to substitute highly sensitive empirical data with opinion-based information. Subjective databases have been used successfully in health care systems to guide resource allocation or medical intervention when empirical data are either limited or inaccessible (Von Winterfeldt and Edwards 1986, Gustafson et al. 1992, Bravington 1996). An added benefit to opinion-based surveys is that geopolitical boundaries are less of an obstacle. This is an important corollary for ISA analyses because Maine shares much of its water, climate, industry affiliations, and pathogens with neighboring New Brunswick.

We constructed an interview composed of about 45 minutes' worth of questions on perceived risks of different management practices and environmental conditions on ISA occurrence. We are querying a panel of experts that includes veterinarians, site and general managers, regulators, and researchers with direct personal experience in the 2001–02 outbreaks. We ask that they base answers to our questions solely on their experience rather than on information gleaned from the literature or scientific meetings. Questions are asked in a manner that provides numerical estimates for the relative importance of perceived risks (Von Winterfeldt and Edwards 1986, Gustafson et al. 1992).

Our numerical estimates are based on percentages of a set of sites which, in the expert's experience, are known to have particular characteristics. We ask them to think about 10 ISA-positive sites and 10 ISA-negative sites from a certain region. Of these sites, we ask them, How many would you classify as strong current sites (or moist feed sites, or multigeneration sites, etc.)? The answers form a likelihood ratio expressing a measure or degree of perceived relative risk. For example, the answers that two ISA-positive sites and four ISA-negative sites use moist feed would form a likelihood ratio of 2:4 in favor of the apparent protective effect of moist feed.

An iterative interview process (Gustafson et al. 1992) is followed wherein experts are asked to review and defend any unusual answers relative to the averaged scores of the other participants. Note-worthy position statements are circulated to all participants, who are given a chance to respond or edit their original answers. Final answers, then, are based on personal reflection followed by group revision and confirmation. Group statistics are reported as medians and quartiles of the revised likelihood ratios and represent the relative magnitude and variability of perceived risks. In this way experts can discuss their experiences in generalities rather than specifics and still address pertinent questions about farm-to-farm transmission.

From this process we will gain a scaled-back set of risk factors deemed important by the majority of those surveyed. The results of this subjective process incorporating Canadian and U.S. expertise can provide practical guidance for future disease control and international conciliation efforts. We also hope that joint agreement on the probable importance of a few select factors in the dissemination of the 2001–02 outbreak will encourage all involved to pursue empirical confirmation of the findings.

### **Coded Empirical Data**

We hope to confirm some of the findings from our opinion-based research with an evaluation of a select portion of empirical data, focusing on just those factors specified as high priority by the expert panel. Although disclosure of site locations would reveal company identity, other data can be coded by a third party for site names and sequenced by dates of clinical infection. Risk factors that do not require spatial analysis can be evaluated in this fashion. By limiting the volume of data required to a short list of select factors and by blinding USDA to the most sensitive information, empirical data collection efforts will not be quite as intrusive.

### **Conclusion**

By using a combination of subjective and coded empirical data, we hope to access industry data that would otherwise be considered too sensitive to share. Information obtained through these two techniques may help guide management and regulatory decisions until further and possibly less confidential empirical databases are established.

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# The Epidemiology of Infectious Salmon Anemia in Scotland

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**Abstract:** Infectious salmon anemia (ISA) was confirmed in May 1998 in farmed Atlantic salmon at Loch Nevis on the west coast of Scotland. The disease rapidly spread from Argyll to Shetland, which represents almost the entire region over which marine salmon farming occurs in Scotland. However, outbreaks were scattered throughout this area: 36 farm sites were suspected, of which 11 were confirmed out of a total of 343 active sites. Isolates of the ISA virus (ISAV) were genetically identical; therefore, outbreaks were linked by spread and were not due to some widespread environmental factor. The pattern of widely but relatively lightly scattered outbreaks is not that expected from spread through the environment. Instead it reflected

patterns of movements within the aquaculture industry—in particular movements of wellboats that serviced the industry. It can be concluded that movements of live fish in wellboats spread ISA over a large area and that wellboats collecting harvests explained most of the remaining spread. The relationships between the number of vessel visits for these purposes and the suspicion or confirmation of ISA were statistically significant ( $p < 0.002$ ). There was no evidence of spread through the environment, but such contagion may have been significant where sites were located within a few kilometers of each other. In such instances, analysis of wellboat movements could not resolve the specific route of ISA contagion.

## ISA in Scotland: Epidemic Spread

ISA is a List I notifiable disease within the European Union (EU), and a compulsory eradication policy is in force. In May 1998, this orthomyxoviral disease was detected at an Atlantic salmon (*Salmo salar*) farm in Loch Nevis on the west coast of Scotland (Rodger et al. 1998). The disease rapidly spread to marine salmon farms around Scotland, and by August, it was present at sites along 650 km of the Scottish coast, from Argyll to the Shetland Islands (Stagg et al. 2001). The analyzed viral isolates were genetically identical in viral segments 2 and 8 (Cunningham and Snow 2000 and their paper in this volume). Outbreaks were also linked epidemiologically (Scottish Executive Fisheries Research Services 2000, Murray et al. 2002) as described in this paper. The genetic and epidemiologic evidence means that a virulent pathogen was spread between sites (as opposed to a latent pathogen being triggered by environmental factors). The disease is also spread as an infection in other countries, such as Norway (Jarp 1999). This paper discusses the epidemiology behind the spread of ISA in Scotland and its implications for disease control measures.

## Basic Theory of Epidemic Disease

The basic spread of an epidemic disease may be described by dividing the population of hosts ( $N$ ) into three classes (Anderson 1981, Reno 1998): susceptible ( $S$ ), infected ( $I$ ), and removed ( $Q$ , which may represent resistant or dead hosts). For a disease with turnover that is rapid relative to that of the host population (births and deaths not due to the disease), the dynamics of infection can be simplified to fit the following equations as described by Murray et al. 2001a:

$$dS/dt = -\beta SI \quad (1)$$

$$dI/dt = \beta SI - mI \quad (2)$$

$$dQ/dt = mI \quad (3)$$

Parameters are the rate of transmission ( $\beta$ ) and of removal ( $m$ ) of infection (cure or death of host). From equation 2, the disease will have a rate of spread (new cases per case removed) equivalent to

$$R_0 = \beta S/m. \quad (4)$$

If  $R_0 > 1$ , removal of cases of infection is less than the rate at which new cases occur, and an epidemic will progress (Anderson 1981). Thus,  $R_0$  depends on the parameters  $\beta$  or  $m$  and the variable  $S$  (Reno 1998). This analysis illustrates the basic factors behind disease transmission, and it is not intended as an applied model of ISA's spread.

The value of  $m$  can be increased by rapid reporting and culling of infected fish (Reno 1998). Speedy removal of clinically infected fish is one of the

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most important tools for controlling ISA (Scottish Executive Fisheries Research Services 2000).

The appropriate units of  $S$ ,  $I$ , and  $Q$  depend upon the mode of infection transmission (Anderson 1981, Murray et al. 2001b), which may be density dependent (farms  $\text{km}^{-2}$ ) or independent (proportion of farms, so  $N = 1$ ). As the names imply, density-dependent transmission will increase with farm density, whereas density-independent transmission does not do so.

Whether transmission is density dependent or independent, it is theoretically possible to reduce  $S$  by vaccination. However, current vaccines may be ineffective and leave fish capable of carrying the disease (Kibenge et al., this volume); thus, widespread use is not compatible with the current EU policy of eradication.

Different processes of pathogen exchange lead to different patterns of disease transmission (Follett et al. 2002). These processes can be passive exchange through the environment, epidemic spread within wild fish populations that infects farms as a side effect, or vectors that actively transport infection between farms.

Passive exchange through the environment is limited by tidal excursion around farms (Scottish Executive Fisheries Research Services 2000). Only nearby adjacent sites are likely to become contaminated, and the epidemic can be broken by physical separation of farms. Survival of pathogens in blood or other organic material could greatly increase the effectiveness of this form of transmission—especially from slaughterhouses adjacent to farms (Nylund et al. 1994). The expected pattern of spread would be between neighboring farms and would be most rapid at high density of farms or where tidal excursion is large.

A second form of disease spread is among wild fish as a classic epidemic. Such epidemics can spread as waves of constant velocity, as has occurred with epidemics among North Sea seals (Swinton et al. 1998) or Australian pilchards (Murray et al. 2001a). Farms could be infected as the

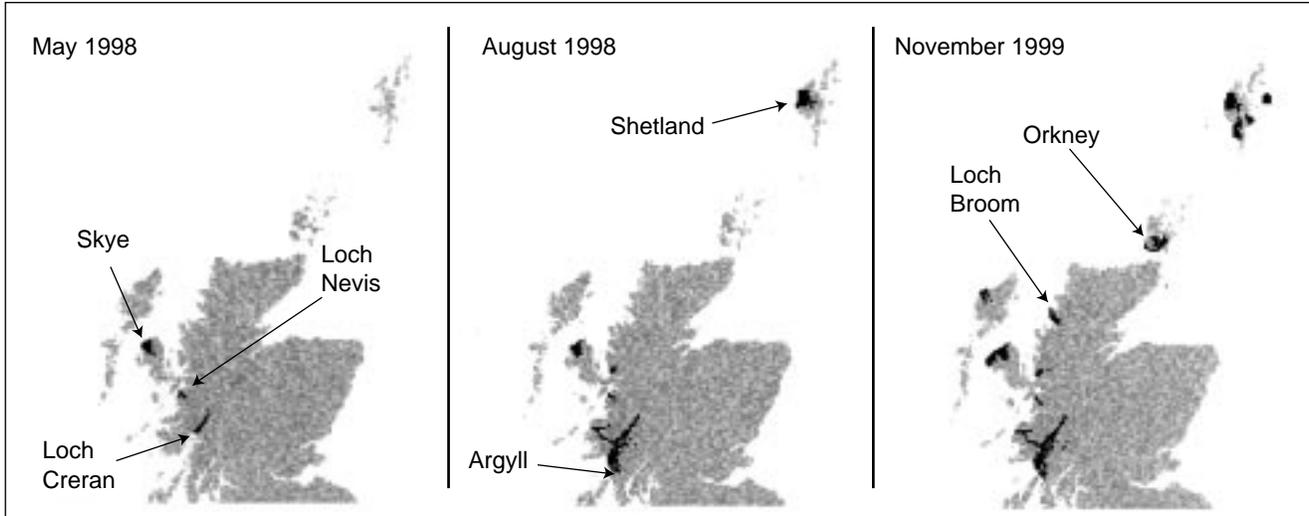
epidemic reaches wild populations in the area. The expected pattern of spread would be as a wave independent of farm density.

Third, natural or anthropogenic vectors may spread disease between farm sites. Natural vectors include birds or fish that travel between farms. Sea trout (*Salmo trutta*), which can act as virus-producing carriers (Nylund and Jakobsen 1995), are more likely to be effective vectors than fish such as saithe (*Pollachius virens*), which do not harbor ISA (Snow et al. 2002). Animals may cover substantial distances: Scottish sea trout were found carrying virulent forms of the ISAv some 84 km from the nearest infected farm (Raynard et al. 2001). There are also many anthropogenic exchanges between farms—particularly via shipping—that may occur over hundreds of kilometers or more. Thus, even though most movements (particularly of fish or birds) are likely to be local, infection may occur at sites distant from previous outbreaks. Vector-transmitted diseases may be only weakly dependent on farm density; that is, where fish farms are far apart, service vessels make long voyages (natural vectors may show more density dependence). Diseases like malaria that are transmitted by insect vectors display such weak density dependence (Anderson 1981).

Viral transmission depends on exchange between different farms and therefore varies between any pair of farms. If a core subset of farms interacts effectively enough such that  $R_{0ij} > 1$ , an epidemic can occur even if the overall  $R_0 < 1$  (Jacquez et al. 1995). As will be seen, a slaughterhouse appears to have played such a key role in the ISA epidemic owing to the large number of its interactions with farms.

## Observed Large-Scale Spread

The observed spread of ISA in Scotland was strongly discontinuous (fig. 1). Outbreaks were rapidly reported over a relatively wide area, and by the end of the summer in 1998, ISA had spread about 500 km north to Shetland and 150 km south to mid-Argyll. This area delineates almost the entire Scottish salmon-farming region. Only later was ISA



**Figure 1**—Spread of control zones around sites suspected or confirmed with infectious salmon anemia (ISA) in Scotland during the epidemic of 1998–99. Figures show the extent of the initial outbreak, spread by the end of summer 1998, and the final extent of the area covered by ISA controls. Locations named in the text are indicated by arrows in the figure where ISA first reached the area.

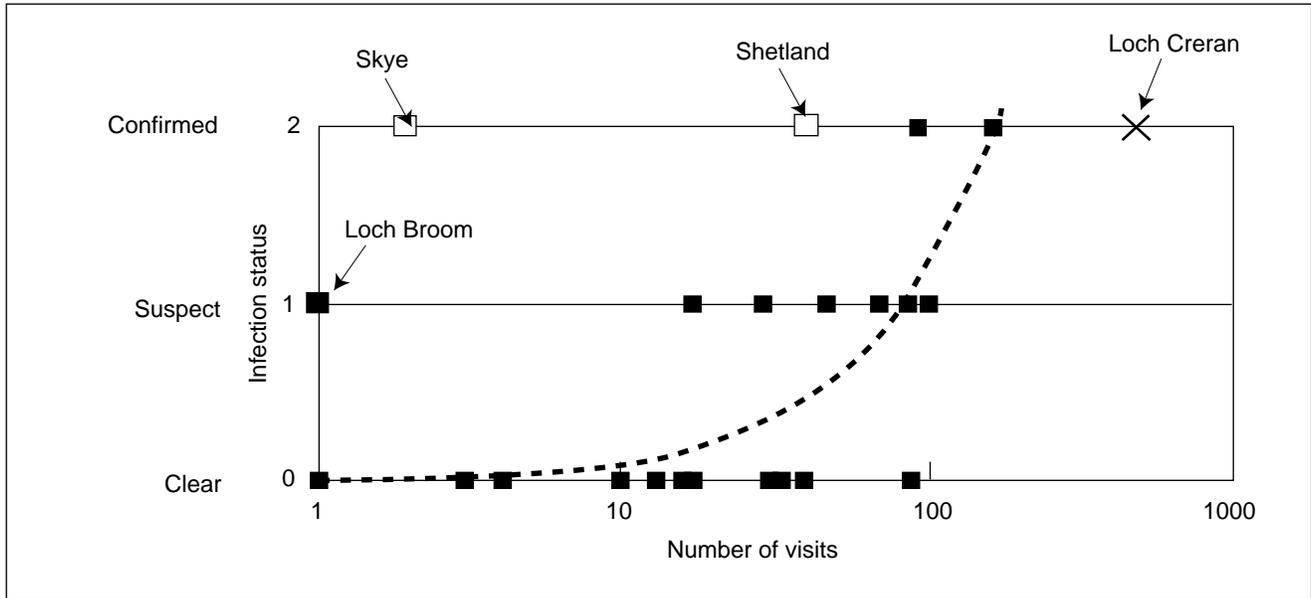
suspected at intermediate locations, such as Orkney, and many areas close to outbreaks did not become infected (Stagg et al. 2001). Out of 343 active marine salmon-farming sites (Stagg and Allen 2001), 36 were suspected of having ISA, but only 11 were confirmed (Stagg et al. 2001). This pattern of a relatively small number of outbreaks scattered throughout the salmon-farming areas implies that there was a highly effective mechanism for long-distance dispersal that was not much more effective over short distances, i.e., vectors.

This observed pattern of spread indicates that vectors transported infection over large distances. The very large distances separating ISA outbreaks strongly suggested anthropogenic exchanges; critical data were available on movements of wellboats servicing the salmon farms. Records were available for the 12 months preceding the epidemic for the 1,558 visits to fish farms made by the 4 wellboats that serviced the Loch Creran processing plant. Individual farms are not always identifiable from vessel records, which were generally grouped by

area. Areas not visited by these wellboats were not included in the analysis. The processing plant at Loch Creran is also excluded because it was qualitatively different from other sites (Murray et al. 2002).

If ISA was confirmed in at least one site within an area, it was given a disease status of 2. If there was suspected but no confirmed ISA in an area, it was assigned a disease status of 1. If there was no suspected or confirmed ISA within the area, it was classified as 0. Definitions of confirmed and suspect sites are discussed elsewhere (Stagg et al. 2001, Murray et al. 2002).

Outbreaks of ISA showed a very clear link to the number of visits by wellboats ( $p = 0.0015$  by linear regression), but some areas developed ISA after relatively few wellboat visits (fig. 2), which produced some scatter in the relationship ( $r^2 = 0.23$ ). Particularly outstanding are Skye and Shetland; however, these areas received shipments of live fish directly or indirectly from the original site of infection at Loch Nevis (Stagg et al. 2001). This is a highly



**Figure 2**—ISA disease status of areas versus the number of wellboat visits. Infection status is 0 if there was no infection, 1 if infection was suspected, and 2 if infection was confirmed. The processing plant at Loch Creran is shown as an X. Sky and Shetland (large hollow squares) were infected by fish transferred from Loch Nevis. Loch Broom is shown as a large filled square. Regression line shown (small squares only) is  $0.012 \times \text{visits}$ ,  $r^2 = 0.66$ , and  $p = 0.000004$ .

effective means of spreading diseases (Stewart 1990). Loch Broom is also unusual because infection was only suspected in June 1999; thus it was not part of the initial spread. Exclusion of these areas from the analysis produced a greatly improved fit ( $p = 0.000004$ ,  $r^2 = 0.66$ ).

Because different routes of transmission may have different efficiencies, the vessel visits have been subdivided into three categories: movements of live fish, harvest collection, and other visits (e.g., goods delivery). Multiple regression has been carried out (using the data analysis package in Microsoft Excel) for infection status against these three categories of vessel visits and distance from the processing plant. The regression was performed on three different data sets (table 1). Data sets were defined as follows: I. All data except Loch Creran, II. Exclusion of other areas known to have directly received infected fish (Skye and Shetland) and Loch Broom, III. Only sites

within 50 km of Loch Creran, which excludes Skye, Shetland, and Loch Broom, have been included.

Large-scale spread of infection is accounted for by wellboat visits delivering live fish or those concerned with harvest collection. All the analyses showed that there was no relationship between general-purpose visits or distance from the processing plant with infection status. Analysis of all data (I), excluding only Loch Creran, gives a good explanation of the infection status ( $r^2 = 0.43$ ) with significant specific relationship to movements of live fish and harvest collection. When the areas that had become contaminated via shipments of infected fish from Loch Nevis and Loch Broom (II) are excluded, the remaining infections are unrelated to live fish transport. The relationship with harvest collection, however, is now very significant ( $p = 0.001$ ), and explanation of the disease status of remaining areas is very good ( $r^2 = 0.69$ ). A similar pattern occurs for

**Table 1—Multivariate analysis results<sup>1</sup>**

	$r^2$	Harvest	Fish movement	General	Distance
I	0.43	<b>0.03</b>	<b>0.09</b>	0.65	0.59
II	0.69	<b>0.001</b>	0.43	0.28	0.29
III	0.62	<b>0.05</b>	0.41	0.82	0.44

I: Excluding Loch Creran only.

II: Excluding Loch Creran, Loch Broom, Skye, and Shetland.

III: Within 50 km of Loch Creran (excluding Loch Creran).

<sup>1</sup>Values of  $r^2$  and  $p$  of significance of relationships of infecton status obtained for different visit types and distance from processing plant (statistically significant  $p$  values are in bold).

areas that were within 50 km of the processing plant (III) but is far less significant ( $p = 0.05$ ) because there were relatively few such data cases to analyze.

## Local Spread

Because analysis of wellboat movements does not pinpoint individual farms, it was not useful for analysis of transmission at distances of a few kilometers or less. The presence of infection at farms, or especially at slaughterhouses, within 5 km was shown to be a significant risk factor for contagion of ISA into other sites (Jarp and Karlsen 1997), particularly if slaughterhouses were inadequately disinfected. Local spread of virus with blood when slaughter occurs at the farm may be important (Munro et al. 2003). Sea lice may also transmit virus over short distances (Jarp and Karlsen 1997). Additionally, local scavenging fish or bird populations may act as vectors that are effective only at scales of a few kilometers or less.

Eradication of ISA from the United Kingdom involved culling of entire farm sites, whereas in countries in which ISA has not been eradicated, only cages housing infected fish were culled. The link between cage-only culling and unsuccessful eradication efforts may indicate that ISA can be effectively transmitted between cages. There was also probable transmission between sites at a location such as those in Loch Nevis (Stagg et al.

2001). Intersite spread of disease gave evidence of effective transmission at scales of tens to thousands of meters.

## Discussion

The spread of ISA in Scotland provides evidence for the anthropogenic spread of ISAv. A clear, statistically significant picture of the emergence of ISA is available: shipments of live fish from Loch Nevis spread ISA around the country, and then movements from the harvest processing plant at Loch Creran spread the disease along the west Highland coast. Similar processes probably were involved in Shetland, where ISA spread across the archipelago. At a local scale, ISA may have spread through the water, particularly in the presence of blood and perhaps parasites.

The spread of ISA in Scotland has strong parallels to the spread of foot-and-mouth disease (FMD) in the United Kingdom in 2001. The virus causing that disease was also spread over large distances by the movement of live animals and spread between neighboring farms by a variety of pathways (Follett et al. 2002).

An important tool that minimizes ISA emergence is the control of fish transfers between seawater sites, and this is accepted in the industry code of practice for control of ISA. Control of local spread is also achieved by separating farms into management areas, which are based on the parameters of tidal excursion distances (Scottish Executive Fisheries Research Services 2000).

Evidence suggests that ISA was spread by harvesting operations. Clearly, harvesting is an essential part of salmon farming; however, harvesting practices should be designed to reduce the risks (Munro et al. 2003). Local slaughter at the farm is likely to present risks to neighboring farms, whereas slaughter at a central slaughterhouse may risk widespread transmission. If fish are delivered directly into the processing plant for immediate slaughter and effluent is thoroughly disinfected, the risk of transmission can be minimized.

The virus causing ISA in salmon can be carried by sea trout (Nylund and Jakobsen 1995), and the virulent virus has been isolated from wild Scottish sea trout far removed from any farms (Raynard et al. 2001). Should virulent ISAv become established in wild populations, eradication may become impossible.

Although ISAv has been found to be widespread but at low prevalence in Scottish wild salmonids, in most cases it does not appear to cause disease or be associated with ISA on farms (Raynard et al. 2001). Genetic evidence supports independent origins for ISA in Canada and Norway (Blake et al. 1999). These facts suggest a reservoir of virus from which virulent strains occasionally emerge and, if true, ISAv may resemble the closely related influenza virus (Webster 1998; Cunningham and Snow, this volume). Increased time or larger numbers of wild-type viruses will increase the opportunity for virulent virus to evolve. Stressed fish maintained at the same site for generations without fallowing will provide an environment where disease emergence is likely. The Loch Nevis site, at which ISA emerged, had a chronic (non-ISA) disease history and overlapping generations, and thus suitable conditions for ISA emergence existed (Stagg et al. 2001).

Scottish ISA may have originated independently, or it may have been imported from Norway (Cunningham and Snow 2000). Although not identical to any known strain, Scottish ISAv was very similar to Norwegian ISAv isolates (Cunningham and Snow, this volume), suggesting importation. Scotland does not allow imports of live salmonids or their eggs from Norway, but nevertheless there are strong links between the two countries' industries. Much of the Scottish industry is Norwegian owned, and several of the wellboats used were built in Norway. Eviscerated Norwegian and Faroe Island salmon are processed in Scotland, and on one occasion this involved fish from an ISA-suspect Faroese farm (Pauline Munro, personal communication). A formal risk assessment is being conducted by Fisheries Research Services inspectors, enabling identified risky imports to be controlled under World Trade Organization rules

(Follett et al. 2002). The risk of ISA's returning to Scotland, by either importation or viral mutation, remains serious.

A range of environmental and management factors will result in differences in ISA epidemiology between countries. It appears that ISA was successfully eradicated through a policy of culling in Scotland. In Norway, however, ISA is well established, and eradication has not been achieved. In Norway, virulent ISAv may be established in a wild reservoir. In salmon-farming areas of Atlantic North America, strong tidal currents and short distances between farms may make local transmission mechanisms more important for ISA's spread. In Pacific North America, a range of different salmonid species may result in a complex epidemiology should an outbreak occur there.

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# Improved Diagnosis of Infectious Salmon Anemia Virus by Use of a New Cell Line Derived From Atlantic Salmon Kidney Tissue

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**Abstract:** Infectious salmon anemia (ISA) is an important viral disease of cultured Atlantic salmon (*Salmo salar*) in Norway, Scotland, Canada, Denmark, Chile, and more recently, the United States. Current diagnostic methods include isolation of the ISA virus (ISAv) in cell culture and detection of specific transcriptional products by reverse-transcriptase–polymerase chain reaction (RT–PCR) and microscopic observation of viral proteins in affected cells by staining with fluorescently labeled specific antibodies. We

compared the SHK–1 cell line to another cell line also developed from Atlantic salmon pronephros, the Atlantic salmon kidney (ASK) cell line. Although ISAv can be cultured in both cell lines, we found ASK cells to be more desirable for routine laboratory use. Not only are ASK cells easier to maintain, they also display a distinct viral-induced cytopathic effect (CPE) that provides the researcher and diagnostician with a new tool to detect and quantify ISAv levels in fish tissues.

## Introduction

Infectious salmon anemia (ISA) is a viral disease responsible for severe economic losses in the Atlantic salmon aquaculture industry. Epizootics have been reported in Norway since 1984 (Thorud and Djupvik 1988), and recently the disease has been diagnosed in Scotland (Rodger et al. 1998, Stagg et al. 1999, Turnbull 1999), Canada (Mullins et al. 1998, Bouchard et al. 1999, Lovely et al. 1999), the United States (Bouchard et al. 2001), Chile (Kibenge et al. 2001), and the Faroe Islands (Anonymous 2000).

Prior to 1995, routine in vitro isolation and propagation of the ISAv was not possible because existing continuous fish cell lines did not appear to support replication of the virus. This hampered characterization of the virus and, consequently, the development of diagnostic methods. Dannevig et al. (1995) reported that ISAv could be isolated using SHK–1 cells, a cell line derived from the Atlantic salmon (*Salmo salar* L.) pronephros. The SHK–1 cell line is now used routinely to culture ISAv in diagnostic and research laboratories. This ability to propagate the virus has proved immensely important, as evidenced by the subsequent development of a monoclonal antibody to ISAv (Falk and Dannevig 1995) and RT–PCR diagnostic techniques (Mjaaland et al. 1997) that can aid in the detection of ISAv infections. More recently, it has been determined that

the long-established CHSE–214 cell line (Lannan et al. 1984) from chinook salmon (*Oncorhynchus tshawytscha*) embryo (Bouchard et al. 1999, Lovely et al. 1999) and the AS cell line (Nicholson and Byrne 1973) derived from the visceral organs of Atlantic salmon (Sommer and Mennen 1997) also supported the replication of ISAv. However, viral production and cytopathic effect (CPE) in these cell lines is too variable for routine use.

Interestingly, Falk et al. (1998) reported many of the same problems when using the SHK–1 cell line, including low yields of virus and poorly defined and slow developing CPE. Kibenge et al. (2000) also reported that not all strains of ISAv could replicate in CHSE–214 cells, and among those which did replicate ISAv, viral titers were lower than those produced in SHK–1 cells. The North American ISAv isolate used in this study is known to cause CPE in the CHSE–214 cell line; however, neither the North American nor European strains of ISAv produced CPE in a pilot study using the CHSE–214 cell line at the Western Fisheries Research Center.

Wergeland and Jakobsen (2001) reported the development of a new cell line established from Atlantic salmon head kidney leukocytes designated the TO cell line. In developing the TO cell line, the authors hoped to provide researchers and diagnosticians with a robust, highly ISAv-sensitive, and stable cell line. The cell line was passed more than 150 times without changes in morphology, growth characteristics, or viral yields. However, availability of the TO cell line is currently very limited because of patent considerations.

Devold et al. (2000) also reported the isolation of an Atlantic salmon head kidney cell line (ASK) that

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is susceptible to ISAv and, following infection with the virus, displays a distinct CPE (cell rounding and detachment from the substrate) in just 7 to 8 days. By September 2002, the cell culture had been successfully passed 30 times.

The SHK-1 cell line remains the most commonly used cell line for the clinical diagnosis of ISA despite the fact that ISAv production can be very low and the weak CPE has limited diagnostic value. Therefore, it was of interest to find a cell line that could easily be adopted into a laboratory's existing cell-culture routine and that also produced high ISAv titers as well as a distinct and complete CPE. The ASK line was chosen for comparison with the SHK-1 cell line, and results suggested that the ASK cell line is superior for use when ISAv research studies or routine diagnosis requires viral isolation.

## Materials and Methods

### Cell Lines

Low-passage number SHK-1 cells were obtained from the Central Veterinary Laboratory (Oslo, Norway) and high-passage number SHK-1 cells were obtained from Micro Technologies, Inc. (Richmond, ME). The SHK-1 cells were routinely cultured in Leibowitz's L-15 medium supplemented with fetal bovine serum (FBS; 10-percent volume to volume [v/v]), 4 milli-molar (mM) glutamine, 100 units penicillin, 100 µg streptomycin, 0.25 µg amphotericin B and 2-mercaptoethanol (0.1 mM). Low-passage ASK cells were obtained from the University of Bergen, Department of Fisheries and Marine Biology, Section for Fish Health, and were propagated using the same media formulation as for the SHK-1 cells (L-15 with 10-percent FBS), with the exception of 2-mercaptoethanol, which was excluded. Both cell lines were incubated at 15 °C and were subcultured every 10-16 days at a split ratio of 1:2.

### North American and European Virus Strains

The ISAv strain CCBB was isolated by Micro Technologies, Inc., from an ISA outbreak in Atlantic salmon in Back Bay, New Brunswick (Canada). The Bremnes strain of ISAv was isolated from an outbreak in Bremnes (Norway) by the University of Bergen, Department of Fisheries and Marine Biology, Section for Fish Health.

### Virus Production

Routine production of both strains of ISAv was done using the SHK-1 cell line. Following media removal, 75-cm<sup>2</sup> flasks of cell monolayers were inoculated with either 100 µL ISAv CCBB or ISAv Bremnes. The virus was allowed to adsorb to the SHK-1 cells for 30 min, after which Leibowitz's L-15 medium supplemented with 5-percent FBS v/v, 4 mM glutamine, 100 units penicillin, 100 µg streptomycin, 0.25 µg amphotericin B, and 2-mercaptoethanol (0.1 mM) was added. The SHK-1 monolayers were held at 15 °C until maximum CPE was observed. The pooled culture fluids for each strain were stored in 1.0- or 10.0-mL aliquots at -80 °C.

### Virus Titrations

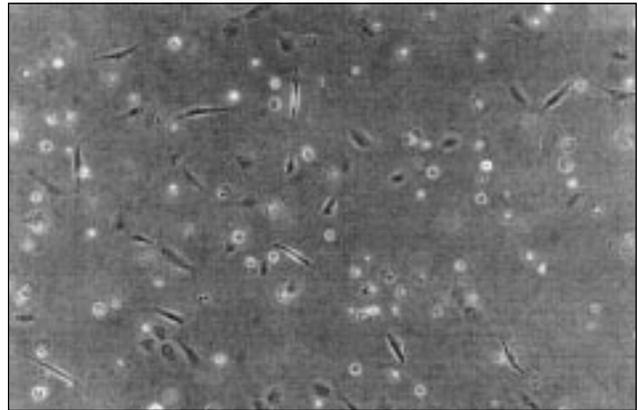
The sensitivity of the two cell lines to ISAv was determined by titrating virus supernatants in the ASK cell line using the 50-percent tissue culture infectious dose (TCID<sub>50</sub>) viral assay with 8 replicate wells per dilution in 96-well tissue-culture microplates. Cell cultures were drained, and each well received 100 µL of a log<sub>10</sub> viral dilution prepared in Leibowitz's L-15 medium supplemented as before and containing no FBS (L-15 0). Following virus inoculation, cultures were incubated at 15 °C in plastic containers supplemented with a blood gas mixture until maximum CPE was observed (8 to 28 days). Viral titers were calculated using the standard endpoint dilution method described by Reed and Muench (1938).

To compare the SHK–1 and ASK cell lines, 75-cm<sup>2</sup> cell-culture flasks of confluent SHK or ASK cells were inoculated with 100 µl of ISAv, either CCBB or Bremnes, as before. Cell cultures were monitored regularly for the presence of CPE, which included a daily photographic record of each culture. After 8 to 28 days, or when maximum CPE was observed, the cell-culture supernatant was removed and serial log<sub>10</sub> dilutions were prepared in L–15(0) to determine the virus titer. Whereas virus titers for each experimental culture were measured on both cell lines, only results from titrations on the ASK cell line are reported.

## Results and Discussion

The virus titer of the pooled culture fluids was determined to be 10<sup>8</sup> TCID<sub>50</sub>/mL for both ISAv stocks. Final endpoint titers of ISAv were comparable in SHK–1 and ASK cells on the basis of titrations using the ASK cell line (table 1). Accurate measurement of virus titers from SHK–1 cells was impossible because of a lack of distinct CPE. Falk et al. (1997, 1998) developed an immunofluorescence assay that can be adapted to quantify viral levels within the SHK–1 cell line in the absence of CPE, but we did not believe a

serological assay was appropriate for this comparison. ISAv induces a rapid detectable CPE in the ASK cell line (fig. 1) consisting of complete lysis and detachment from the substrate within 14 days. In contrast, the CPE caused by ISAv in SHK–1 cells is not definitive. In the first 14 days after ISAv inoculation, some SHK cells detach from the substrate. These possibly represent a subpopulation of the cell line permissive to viral replication, although the monolayer of fibroblastlike cells appears to be maintained (fig. 2). Up to 25 days after ISAv



**Figure 1**—Cytopathic effect (CPE) of ISAv in ASK cells consisting of lysis and detachment of the cells from the substrate. Photograph taken at 14 days after infection at 100x magnification. (This and all other photographs were taken by coauthor James R. Winton.)

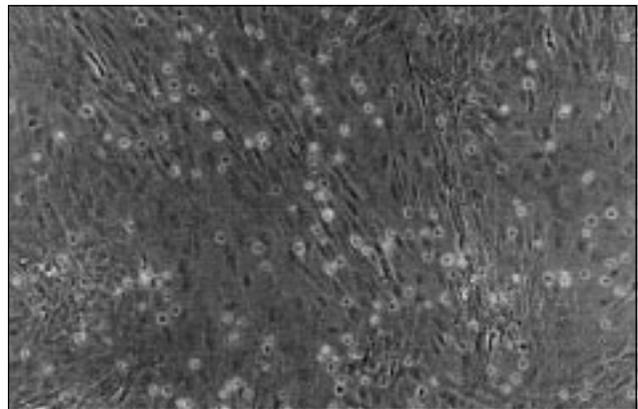
**Table 1**—Comparison of virus titers in the North American and European strains of ISAv that were grown in either the SHK–1 or ASK cell lines

ISAv strain	Virus titer	
	Cell line	TCID <sub>50</sub>
Bremnes <sup>1</sup>	SHK–1	10 <sup>6.4</sup>
CCBB <sup>2</sup>		10 <sup>5.7</sup>
Bremnes	ASK	10 <sup>6</sup>
CCBB		10 <sup>6.1</sup>

*Note:* Confluent monolayers of either ASK or SHK cells were inoculated with either the CCBB or Bremnes strain of ISAv. After 8 to 28 days, or when maximum cytopathic effect was observed, cell-culture supernatant was removed and titered on ASK cells.

<sup>1</sup>European.

<sup>2</sup>North American.



**Figure 2**—SHK–1 cells infected with ISAv. CPE consists of some cell rounding and detachment from the substrate. Photograph taken at 100x magnification 14 days after infection.

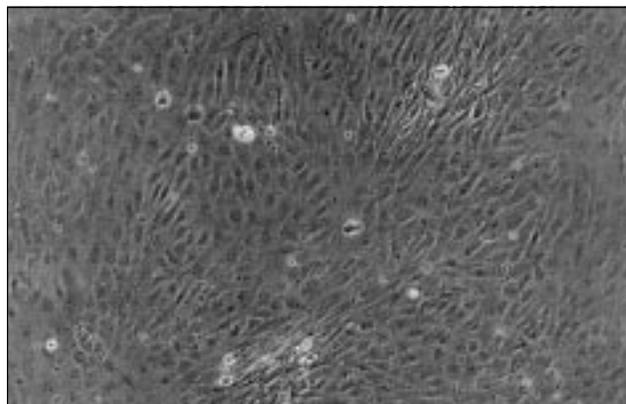
inoculation, we observed morphological changes in the SHK cells, such as vacuolization, that could be attributed to factors other than viral replication (fig. 3).

The ASK cell line resembles epithelial cells, and at the Western Fisheries Research Center has retained that morphology with each subculture (fig. 4). The SHK-1 cell line has been partially characterized, and the cells were found to resemble macrophages (Dannevig et al. 1995). However, morphologically, the cell line appears to be a mixed population consisting primarily of fibroblastlike cells, but also containing a small population of cells resembling leukocytes (fig. 5). The role of each cell type in ISAv replication is poorly understood. However, the number of leukocytelike cells in a culture declines with each passage. To avoid complete loss of this subpopulation, fresh cells from frozen stocks at a low passage must be placed into culture at regular intervals.

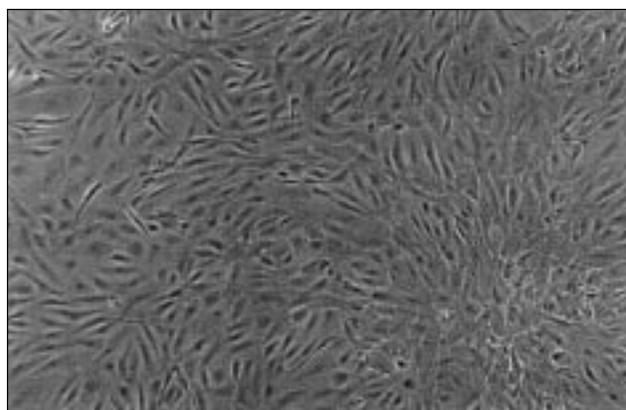
Upon receipt of the SHK-1 cell line, it was necessary to acclimatize the cells using a conditioned medium. Once cells were transferred to growth medium L-15(10), they exhibited poor growth. Growth was regained only after the cells were placed in a medium containing Biowhittaker Australian FBS. At no time did we feel it was necessary to use a conditioned medium when culturing the ASK cells. Although the ASK cells appear to grow faster in a medium containing Biowhittaker Australian FBS, the cell line is not dependent on this serum supplement for growth.

Use of the SHK-1 cell line requires careful attention to culture conditions. For example, the SHK-1 cells appear to be sensitive to cell density as subculturing either before or after the cells reach 100-percent confluence can result in loss of cultures. In contrast, the ASK cultures appear to be more tolerant to subculture once the cells have passed 100-percent confluence, although like the SHK-1s, subculture at low cell densities can also result in loss of a culture.

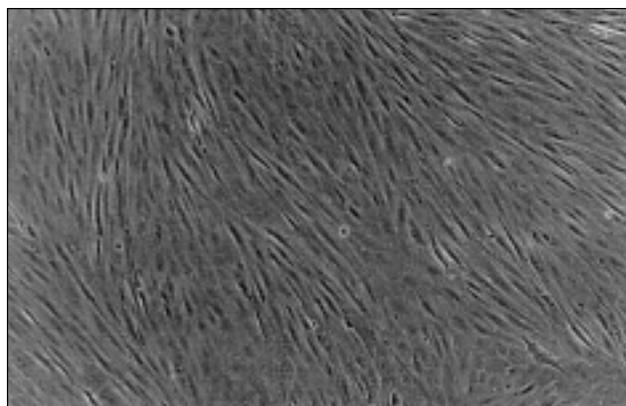
The aim of this study was not to characterize the ASK cell line but to compare it with the SHK-1 cell line for isolation of ISAv. Results suggested that



**Figure 3**—SHK-1 cells infected with ISAv 25 days after infection. The cell monolayer is maintained despite infection. Photograph taken at 100x magnification.



**Figure 4**—A culture of ASK cells consisting of cells resembling epithelia. Photograph taken at 100x.



**Figure 5**—A healthy culture of SHK-1 cells consisting primarily of cells that morphologically resemble fibroblasts and a small proportion of cells resembling leukocytes. Photograph taken at 100x.

the ASK cell line may be superior to the SHK-1 cell line in terms of relative ease of use in the laboratory. More importantly, the presence of clear and distinct CPE by ISAv in ASK cells provides the fish health specialist with an economical method to quantify viral levels. We believe that these characteristics of the ASK cell line also make it appropriate for studying the epizootiology and pathogenesis of ISAv.

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# Evaluation of Infectious Salmon Anemia Diagnostic Tests

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**Abstract:** Infectious salmon anemia (ISA) is a viral disease characterized by lethargy, anorexia, anemia, internal organ damage, and death. Costly control methods used on the east coast of Canada include a surveillance program, early harvest of fish in test-positive cages, and indemnity programs. Test methods used for regulatory decisions include the indirect fluorescent antibody test (IFAT), reverse-transcriptase–polymerase chain reaction (RT–PCR) assay, and virology. Although the diagnostic tests have not been validated, their results are used to make sizable monetary decisions. The objective of this study was to evaluate the sensitivity and specificity of ISA diagnostic tests using data collected by the New Brunswick Department of Agriculture, Fisheries and Aquaculture. Because a “gold standard” reference test for ISA is not available, we used cage status as our distinguishing criterion. A pool of negative fish from farms that had never had the disease and a pool of positive fish from cages that

were experiencing an outbreak defined by greater than 0.05 percent mortalities per day were obtained and assumed to be negative and positive, respectively. We used results from a total of 1,071 (807 negative, 264 positive) fish for this study. On the basis of the test's cutoff value, the sensitivity and specificity for histology ranged from 30 percent to 73 percent and 73 percent to 99 percent, respectively. The IFAT had sensitivities and specificities in the range of 64 percent to 83 percent and 96 percent to 100 percent, respectively. For the RT–PCR assay, sensitivity and specificity were 93 percent and 98 percent, respectively. In test performance evaluation, we factored in the possible clustering of test results by farm that might be attributed to site differences in disease severity or environmental factors. Slight changes in sensitivities and specificities were coupled with widening of the estimated confidence intervals for most cases.

## Introduction

Infectious salmon anemia (ISA) virus (ISAV) has caused disease in farmed Atlantic salmon in New Brunswick since 1996 (O'Halloran et al. 1999). This severe disease, which is characterized by lethargy, anorexia, anemia, death, and internal organ damage (Byrne et al. 1998, Thorud and Djupvik 1998), has occurred sporadically throughout the New Brunswick fish farms in the Bay of Fundy. In 1998, about 22 of the 83 salmon farms were completely depopulated for control purposes (O'Halloran et al. 1999). Costly control methods used on New Brunswick Atlantic salmon farms include a surveillance program, early harvest of fish from test-positive cages, and indemnity programs.

Current industry control programs require ISA testing on dead fish at least every 6 weeks for every farm. Such surveillance results in early slaughter of a cage if there have been 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 virus isolation (VI), the indirect fluorescent

antibody technique (IFAT), reverse-transcriptase–polymerase chain reaction (RT–PCR) assay, and histology on fish tissues (Bouchard et al. 1999; Dannevig et al. 1995a,b; Evensen et al. 1991; Falk et al. 1998; Mjaaland et al. 1997; Simko et al. 2000; Speilberg et al. 1995). Performance characteristics of these tests are unknown, and test results from the same fish are often inconsistent. Although the ISA diagnostic tests have not been evaluated, their results are used to make sizable monetary decisions.

Because performance reliabilities for each of the diagnostic tests were unknown, many tests were performed on tissue from the same fish from 1998 to 2000 by the Provincial government as part of the early surveillance program. Those results were made available to us for evaluation of the diagnostic tests. The objective of this study was to determine the sensitivity and specificity of as many ISA diagnostic tests as possible.

## Materials and Methods

A total of 30,255 test results were available from 8,167 fish. Much of the data was unusable because the disease status of each fish's cage was available only from April 1999 to January 2000. All fish that had diseases other than ISA were removed from the data set. For the purpose of calculating sensitivity

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and specificity, our gold standard for disease status was based on the following criteria: ISA-negative fish came from farms that had no outbreak of ISA during the period, and ISA-positive fish came from cages that were experiencing clinical disease defined by mortalities >0.05 percent per day at the time of sampling.

After we reduced the usable data set, some of the laboratories and tests were further dropped from the analysis because the numbers of samples were too small for statistical analysis. The laboratories included in the study were the Atlantic Veterinary College Diagnostic Lab and Aquatic Diagnostic Services (AVC) in Charlottetown, PE; the New Brunswick Department of Agriculture, Fisheries and Aquaculture laboratory (DAFA) in Blacks Harbour, NB; and the Research and Productivity Council laboratory (RPC) in Fredericton, NB.

All test results were dichotomous or ordinal. Histology was reported on a scale of negative, suspect, and positive. For the sensitivity and specificity, histology data were analyzed in 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 cutoff values: first using 1+, 2+, 3+, or 4+ as a positive result (IFAT 1) and second using 1+ as a negative result and 2+, 3+, and 4+ as a positive result (IFAT 2). The RT-PCR assay and virology test have dichotomous results reported as positive or negative. Given the expense of the virology test, pools of up to five fish were tested as one sample in which all fish in the pool would have a positive result even if only one fish in the pool were positive. The data set was reduced further by identifying the fish that were tested for virology individually (not in a pool). The resulting data was analyzed for sensitivity and specificity.

Sensitivities, specificities, and 95-percent confidence intervals were calculated in two different ways. Initially, test sensitivity, specificity, and 95-percent confidence intervals (exact based on the binomial distribution [Newcombe 1998]) were

calculated from a  $2 \times 2$  table of all fish using the gold standards described above. Secondly, potential test variation between the farms for positive and negative populations was taken into account by using a random effects logistic regression model with the farm as the random effect. Sensitivity was calculated as  $e^y/(1+e^y)$ , in which  $y$  was equal to the constant from the random effects logistic regression model for the ISA-positive population divided by the square root of  $(1 + 0.346 \cdot \sigma^2)$ , in order to obtain a population-averaged estimate (Zeger et al. 1988), where  $\sigma$  was the estimated dispersion of farm random effects. Specificity was calculated using  $1 - (e^y/(1+e^y))$  with  $y$  as above for the ISA-negative population. Confidence intervals for sensitivity and specificity were calculated with the same formulas when substituting the constant by the limits of its confidence interval. The estimated intraclass correlation coefficient (ICC) between samples at the same farm was calculated as  $\sigma^2/(\sigma^2 + 3.29)$  (Snijders and Bosker 1999). Finally, 90-percent prediction intervals giving the range of farm sensitivities and specificities were computed by similar formulas involving  $\sigma$  and the standard error of the constant coefficient. For the virology tests, sensitivities, specificities, and 95-percent confidence intervals were calculated only from the  $2 \times 2$  table using the results from fish tested individually.

## Results

The final data set contained 3,721 test results from 1,071 fish (807 negative and 264 positive). These fish came from 238 different cages and from 23 different farms.

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 1. In general, the sensitivity for histology ranged from 30 percent to 73 percent and 73 percent to 99 percent, respectively, on the basis of the cutoff value. The IFAT had sensitivities and specificities in the range of 64 percent to 83 percent

and 96 percent to 100 percent, respectively. For RT–PCR assay, sensitivity and specificity were 93 percent and 98 percent, respectively. When between-farm variation was taken into account, the estimates changed very slightly.

## Discussion

The farmed Atlantic salmon industry in New Brunswick is currently dealing with a diagnostic testing dilemma. The surveillance program tests many dead 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 tons of nonmarket-size fish and a costly compensation package to the farmer. Because both of these scenarios are unacceptable, the identification of a diagnostic test with high sensitivity and specificity is imperative.

The results of our study found the highest sensitivities and specificities in RT–PCR tests performed by the RPC lab. The RT–PCR test results are usually returned within a few days. Unfortunately,

**Table 1—The estimated sensitivities (Se) and specificities (Sp) for four ISA diagnostic tests in the New Brunswick Atlantic salmon farms**

Test	Number tested	Parameter	Combined estimate (CI) <sup>1</sup>	Population estimate (CI) <sup>1</sup>		Random effect		90% Predicted interval
						estimated ICC <sup>1</sup>	P value	
Histology (positive) <sup>2</sup>	674	Se	73.0 (65.3–79.7)	73.0	(65.5–79.3)	0.00	1.000	66.8–78.3
		Sp	72.5 (68.2–76.4)	72.1	(64.6–79.4)	0.07	0.000	52.8–86.6
Histology (negative) <sup>3</sup>	674	Se	30.2 (23.2–38.0)	29.9	(21.9–39.3)	0.34	0.000	4.7–75.3
		Sp	99.4 (98.2–99.9)	99.4	(98.1–99.8)	0.00	1.000	98.4–99.8
IFAT 1 (DAFA <sup>4</sup> )	871	Se	79.1 (73.2–84.2)	79.4	(69.3–86.9)	0.14	0.011	54.5–94.0
		Sp	95.5 (93.6–97.0)	95.7	(92.4–97.6)	0.11	0.027	89.3–98.9
IFAT 2 (DAFA <sup>4</sup> )	871	Se	64.4 (57.8–70.7)	64.4	(58.0–70.4)	0.00	1.000	59.0–69.5
		Sp	99.9 (99.1–100.0)	99.8	(98.9–100.0)	0.00	1.000	99.2–100.0
IFAT 1 (RPC <sup>5</sup> )	473	Se	82.7 (69.7–91.8)	82.7	(70.0–90.7)	0.00	1.000	72.3–89.8
		Sp	98.3 (96.6–99.3)	98.0	(91.2–99.6)	0.31	0.070	90.3–99.9
IFAT 2 (RPC <sup>5</sup> )	473	Se	73.1 (59.0–84.4)	73.6	(56.7–85.6)	0.05	0.313	52.4–88.2
		Sp	99.8 (98.7–100.0)	99.8	(98.3–100.0)	0.00	1.000	98.8–100.0
RT–PCR	948	Se	92.6 (88.2–95.7)	93.2	(86.2–96.7)	0.10	0.103	82.8–98.1
		Sp	98.1 (96.8–99.0)	96.7	(91.0–98.8)	0.48	0.000	84.5–100.0
Virology (AVC <sup>6</sup> )	21	Se	No samples	N/A	N/A	N/A	N/A	N/A
		Sp	100 (83.9–100.0)	N/A	N/A	N/A	N/A	N/A
Virology (RPC <sup>6</sup> )	72	Se	66.7 (9.4–99.2)	N/A	N/A	N/A	N/A	N/A
		Sp	98.6 (92.2–100.0)	N/A	N/A	N/A	N/A	N/A

<sup>1</sup> CI = confidence interval; ICC = intraclass correlation coefficient.

<sup>2</sup> Suspects were considered positive.

<sup>3</sup> Suspects were considered negative.

<sup>4</sup> New Brunswick Department of Agriculture, Fisheries and Aquaculture.

<sup>5</sup> Research and Productivity Council.

<sup>6</sup> Atlantic Veterinary College.

this test's expensive price (\$55 Canadian per fish) may limit its practical usefulness in the industry. The quickest and cheapest test by far is the IFAT. Unfortunately, this test's sensitivity is, at best, 83 percent. Therefore, 17 percent of the truly positive fish appear as false negatives. Histology did not perform very well as an ISA diagnostic test, but this test does have two advantages: it is inexpensive, and there is the potential of diagnosing a concurrent disease. Performance evaluation of virology was difficult because most samples were pooled for this test. The final evaluation was made only on fish that were tested individually. The small number of fish tested made it impossible to evaluate the random effects of the site. Although the specificity of the virology test is excellent, the sensitivity was poor for RPC's virology test and was not evaluated for AVC's virology test due to an insufficient number of samples. An advantage of virology 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 (Dannevig et al. 1995b).

Although we have estimated the sensitivities and specificities of these diagnostic tests, a critical review of the methods should be discussed. 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 (Brenner and Gefeller 1997). 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. Fish with other types of disease may cross-react with the tests, causing false positives and a subsequent reduced specificity. Because the data were trimmed down significantly to identify obviously diseased and disease-free fish, test performance will appear better than it would have been had the test been applied to the whole population.

Conversely, the sensitivity of the virology test may have been falsely lowered. The DAFA lab pooled tissue samples from one to five fish. Fish in a pool usually came from the same cage. If there were

five fish in a pool, the cage probably had high mortalities and advanced disease. If there was only one fish in a pool, there was probably only that one dead fish in the cage. Therefore, it is very unlikely that the fish in that cage had advanced clinical illness. These fish might have been infected but might not have had sufficiently abundant live virus to create a cytopathic effect on the cell culture easily, which is the endpoint of the virology test.

The random effects model was used to account for fish from one farm being more alike than fish from different farms. This model takes into account the extra variation between farms. In addition, the model provides prediction intervals for the sensitivity (or specificity) of the test used on fish from a new farm from New Brunswick. When extra farm variation 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 genetics, geography, age, and management (feed, handling, sea lice, hygiene, etc.). A hypothetical scenario might be a strain of Atlantic salmon with improved resistance to ISAv that might not replicate enough virus to yield a positive result on the IFAT test. This would result in an increase in false-negative tests for fish from farms with similar genetics. A geographic hypothetical example might be dead fish that come from more remote farms and are not processed as quickly as dead fish from local farms. As dead 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.

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, RT-PCR measures viral RNA, IFAT measures viral antigen, and histology assesses lesions (Bouchard et al. 1999; Dannevig et al. 1995a,b; Evensen et al. 1991; Falk et al. 1998; Mjaaland et al. 1997; Simko et al. 2000; Speilberg et al. 1995). If RT-PCR is

positive, ISA viral RNA is most likely 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 future outcome of the fish cage using diagnostic tests, test results should be interpreted cautiously.

The method of choosing the gold standards for this study was not ideal; however, it does give an estimation of how the tests are performing. These results will be used as the basis for future studies designed to better estimate the sensitivities and specificities. These studies will include analyses that are not based on a gold standard test (Hui and Walter 1980).

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# Development of a Strain Typing Assay for Infectious Salmon Anemia Virus (ISAv)

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**Abstract:** In 1997, infectious salmon anemia virus (ISAv) was first identified within aquaculture cages of the Bay of Fundy, Canada. The initial focus of surveillance and fallowing of sites with ISA-infected fish has since expanded to include characterization of ISAv isolates within Atlantic Canada. This initiative was adopted to identify discrete variances in virulence of strains and to track disease. Thus, a diagnostic assay was developed for surveillance programs capable of providing quick and reliable results for

ISAv strain identification. The assay combined reverse-transcriptase–polymerase chain reaction (RT–PCR) with denaturing gradient gel electrophoresis (DGGE) to identify and type ISAv based on nucleotide variability within specific gene segments. Following initial standardization with known isolates, DGGE was performed on RT–PCR products to rapidly determine strain identity without need for additional manipulations, such as sequencing or restriction enzyme digestion.

## Introduction

Infectious salmon anemia virus (ISAv) was first identified in Norway in 1984 (Thorud and Djupvik 1988) and later in Canada (Mullins et al. 1998, Lovely et al. 1999, Blake et al. 1999, Bouchard et al. 1999), Scotland (Rodger et al. 1998, Rowley et al. 1999), Chile (Kibenge et al. 2001a), and the United States (Bouchard et al. 2001). Initial focus on ISAv infection in eastern Canada included stringent surveillance efforts and implementation of acute management strategies in an attempt to limit further dissemination of the disease.

Because the number of clinical cases went down drastically, more attention has been recently directed toward the characterization and classification of discrete ISAv isolates. The desire to understand strain variability comes from several directions— anecdotal evidence that some ISAv outbreaks claim more fish than others and observations in our lab that low-level infections exist in nonsalmonid species, such as plaice and haddock (unpublished). Furthermore, it was established that Norwegian isolates include strains that can be differentiated by 11 sequence variants within a highly polymorphic region (HPR) of the viral segment encoding for the hemagglutinin (HA) (Devold et al. 2001). The ability to rapidly differentiate between viral strains will provide basic knowledge on virulence, epidemiology, and host specificity that can facilitate management decisions.

The genome of ISAv consists of eight segments of negative-stranded ribonucleic acid (RNA)

(Mjaaland et al. 1997). Segment 2 is believed to encode a polymerase protein (Krossøy et al. 1999); segments 3 and 4 to encode a nucleoprotein and polymerase, respectively (Ritchie et al. 2001a); segment 6 to encode a HA (Krossøy et al. 2001, Rimstad et al. 2001); and segments 7 and 8 to encode putative nonstructural and matrix proteins (Ritchie et al. 2002, Cunningham and Snow 2000, Biering et al. 2002). Identification of these segments revealed the existence of nucleotide variation among isolates, especially in segment 6 (Krossøy et al. 2001, Rimstad et al. 2001, Kibenge et al. 2001b, Devold et al. 2001, Griffiths et al. 2001). Such genetic variation allows for typing of ISAv isolates.

The identification of variability at the nucleotide level can be achieved by a number of means, including restriction fragment length polymorphism (RFLP) analysis and direct sequencing. However, these approaches can be time consuming and costly. Here we present a method that identifies variation in ISAv isolates utilizing RT–PCR and denaturing gradient gel electrophoresis (DGGE) technologies (Myers et al. 1987). The latter has been used to detect mutations in disease studies (Valero et al. 1994), separation of alleles for sequencing (Aldridge et al. 1998), and profiling of complex microbial populations (Muyzer et al. 1993). The DGGE technique is rapid and inexpensive and types multiple samples simultaneously. Following the amplification of targeted nucleotide regions in RT–PCR, DGGE separates the double-stranded fragments based on their different melting temperatures as dictated by nucleotide sequence. Fragments are run in a linear ascending gradient of chemical denaturant and, upon melting, will be retained at a specific location within the gel. Fragments that differ in sequence, therefore,

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may be identified by comparison to known standards. Here we describe the development of an assay that detects genetically distinct ISAv isolates endemic to the Bay of Fundy, Canada, and provides for the possibility of detecting other strains that may be encountered in the future.

## Material and Methods

### *ISAv Isolates*

New Brunswick ISAv isolates used in this study were isolated from aquaculture Atlantic salmon (*Salmo salar*) in the Bay of Fundy, Canada, using salmon head kidney (SHK) cells (Ritchie et al. 2001b). Previous sequencing of the segment 6 HPR from Canadian isolates indicated that there were two main groups of ISAv in New Brunswick (Kibenge et al. 2001b, Griffiths et al. 2001). Isolates NB280 and NB508 were included in this study as representatives from each group. In addition, an isolate from Norway (Glesvaer), Scotland (Loch Nevis), and Nova Scotia were included as standard reference strains. The following GenBank accession numbers submitted by other researchers make reference to the same New Brunswick isolates as used in this study, the isolate names here having been shortened: AF294870 (NB280), AF294874 (NB508), AF294877 (NB877), AF294871 (NB028), AF294876 (NB049), AF294875 (NB002), and AF294873 (NB458). Note, however, that the sequencing data obtained for some of these isolates in this study does not agree with that submitted to GenBank by other researchers. The 30 sequences generated in this study can be found under GenBank accession numbers AY151789–AY151818.

### *RNA Extraction and RT-PCR*

Total RNA was extracted from cytopathic SHK cells using TRIzol® LS reagent (Invitrogen) according to the manufacturer's protocol. Pellets were suspended in diethyl pyrocarbonate (DEPC)-treated water (20–50 µL) and 2–4 µL used as template in RT-PCR. The RT-PCR reactions were done using Ready-to-Go™ RT-PCR beads (Amersham Pharmacia) as described by the manufacturer. Briefly, RNA was reverse transcribed in a total volume of 40 µL using 2.5 µg of random hexamer primers at 42 °C for 30 min followed by 95 °C for 5 min. The PCR primers were then added to a final concentration of 0.4 µM, the total PCR reaction volume being 50 µL. Primers used in this study were designed from the sequences of segments 6, 7, and 8. Segment 8 primers were based on those found in Devold et al. (2000) and were included as a control because they target a conserved region and ensured that a strictly maintained protocol existed. The same protocol has been used for the surveillance program conducted for the New Brunswick Department of Agriculture, Fisheries and Aquaculture (NBDAFA) since 1998. Segments 6 and 7 primers were selected to flank variable regions after comparing multiple international ISAv sequences in the National Center for Biotechnology Information databank. For segments 6 and 7, multiple primer pairs and RT-PCR conditions (data not shown) were tested for the amplification of all five reference isolates. A 40-bp GC clamp (Myers et al. 1985) was added to one of the primers of each primer set following Winmelt™ (BioRad) analysis to ensure that a very high melting point domain existed in the amplified product. The following tabulation lists the primers selected.

Segment 6: HAFnew (GC clamp)

5'cgccccgccgcgccccgcgcccgtcccgcgcccccgccccgTKGTCAAAGANTTTGACCARACA3'  
and 1414mod 5'ACAGWGCWATCCCAAACCTG 3'

Segment 7: MAF1 (GC clamp)

5'cgccccgccgcgccccgcgcccgtcccgcgcccccgccccgCKGAACAAGGWGGAAAAGTGGT3'  
and MAR1 5'TAGCAAGTTCATCAAGGAAAATG 3'

Segment 8: NBFA3 (GC clamp)

5'cgccccgccgcgccccgcgcccgtcccgcgcccccgccccgGAGGAATCAGGATGCCAGGACG3'  
and RA3 5' GAAGTCGATGAACTGCAGCGA 3'

Final MgCl<sub>2</sub> concentrations in PCR were either 1.5 mM (segment 6 and 7 primers) or 2 mM (segment 8 primers). Conditions for PCR amplification were as follows: 40 cycles of 94 °C for 30 sec, 62 °C for 45 sec, and 72 °C for 1 min 30 sec. After mixing with loading buffer, amplified products were run on 11-percent acrylamide TBE gels (Mini PROTEAN® II, BioRad) at 200 V for 1 h to check for PCR product yield prior to DGGE analysis.

### **Denaturing Gradient Gel Electrophoresis**

DGGE was performed using the DCode Mutation Detection System (BioRad) utilizing 1.5-mm-thick × 15-cm long, 0.5 × TAE gels of varying percentages of urea–formamide mixture (7 M urea–40 percent formamide), 6-percent polyacrylamide (using 40 percent acrylamide–bis solution, 29:1), and a nondenaturing 4-percent stacking gel. During the optimization process, a broad gradient of denaturant was initially used. Based on the observed migration of RT–PCR products, progressively narrower ranges of denaturant were then tested. Electrophoresis was conducted at 80 V, 60 °C for varying amounts of time (12–17 h).

### **Sequence Analysis**

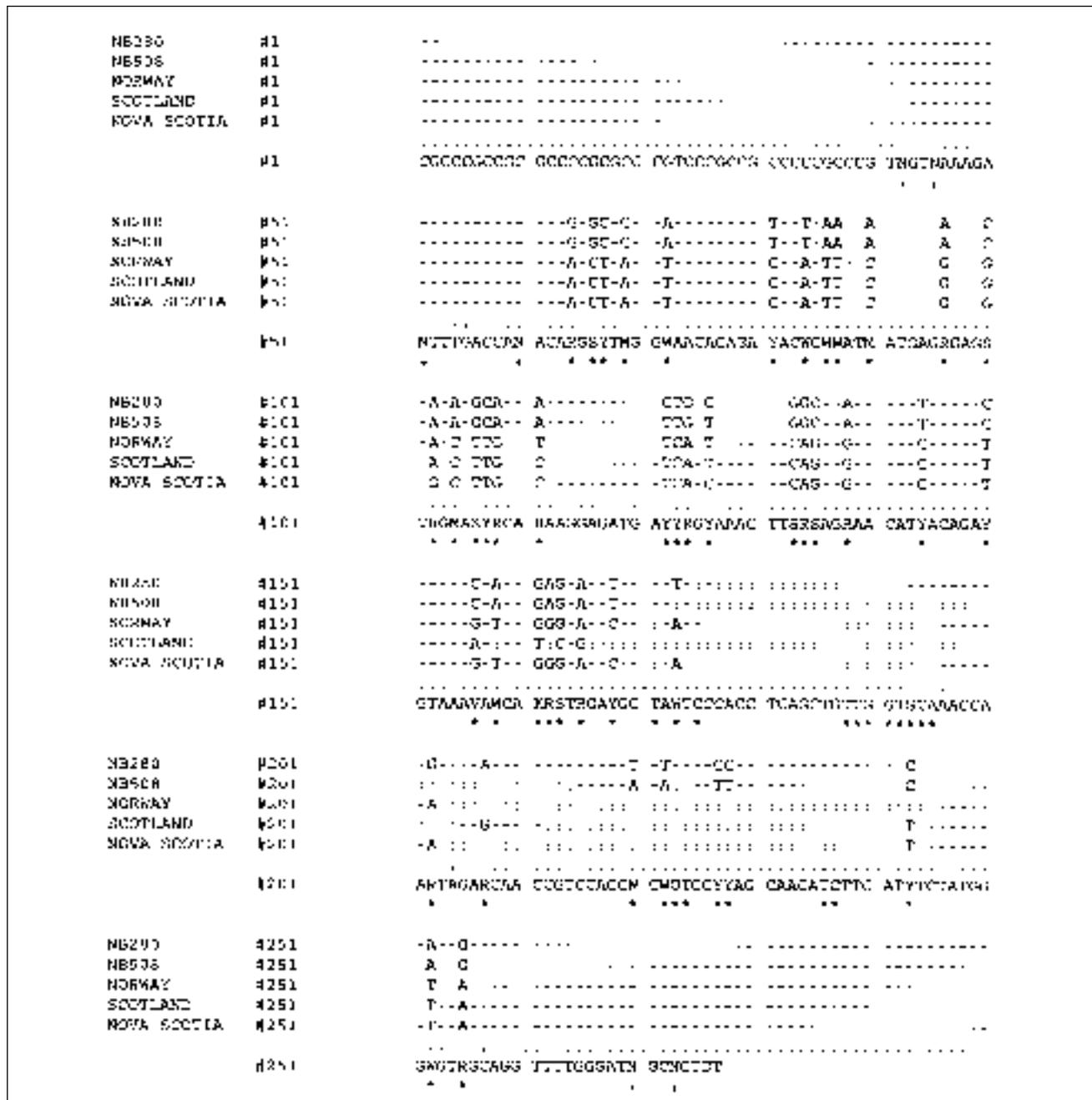
Isolate identification was confirmed by sequencing PCR products. The PCR products were

purified using the Qiaquick® PCR purification kit (Qiagen) and eluted in 50 µL of EB buffer. Three µL of purified PCR product were then added to 3.2 pmol of sequencing primer and 8 µL of Big Dye™ Terminator solution (PE Applied Biosystems) mixed 1:1 with *halfBD*™ sequencing reagent (BioCan Scientific) and cycled 25 times at 96 °C for 10 sec, 50 °C for 5 sec, and 60 °C for 4 min. The total reaction volume was 20 µL. Primers used for sequencing were the same as those used in PCR. Following the cycle sequencing reaction, the mixture was applied to Performa® DTR gel filtration cartridges (Edge BioSystems). The eluted product was heated to 95 °C and snap-cooled on ice. Samples were then electrophoresed on an ABI 3100™ Genetic Analyser and further examined using Sequencher™ (GeneCodes).

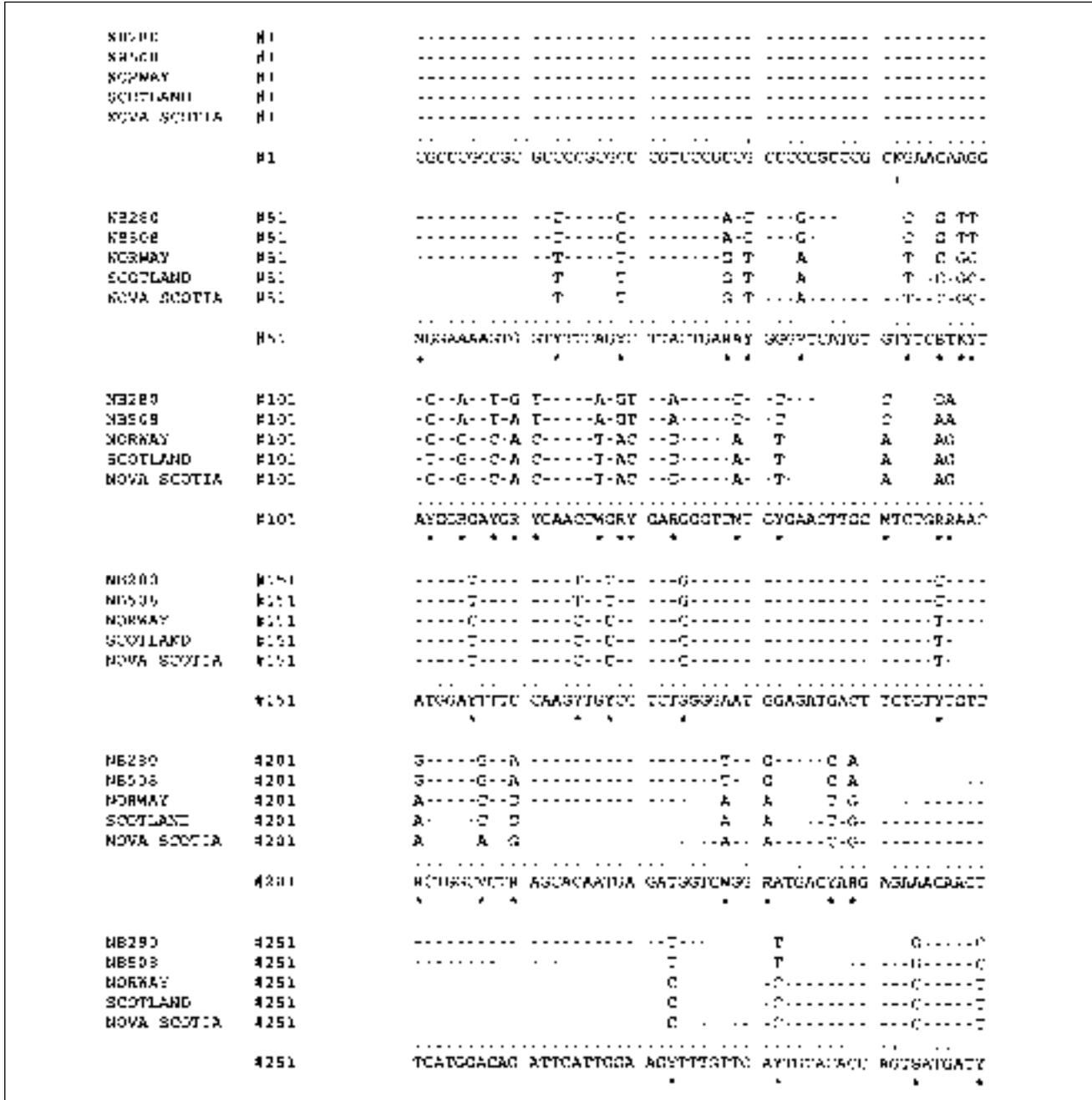
## **Results**

The primer sets tested, including HAFnew (GC clamp)/1414mod for segment 6 and MAF1 (GC clamp)/MAR1 for segment 7, successfully amplified all five of the ISAv standard isolates, as did the segment 8 primers, NBFA3 (GC clamp)/RA3. Due to the presence of insertions/deletions (indels) in the HPR of segment 6, primers HAFnew (GC clamp)/1414mod produced products of variable length in RT–PCR ranging from 217 to 265 bp. Segment 7 and 8 primers amplified a 454 bp and 251 bp fragment, respectively, for each isolate in the panel. The three amplified regions of each isolate were

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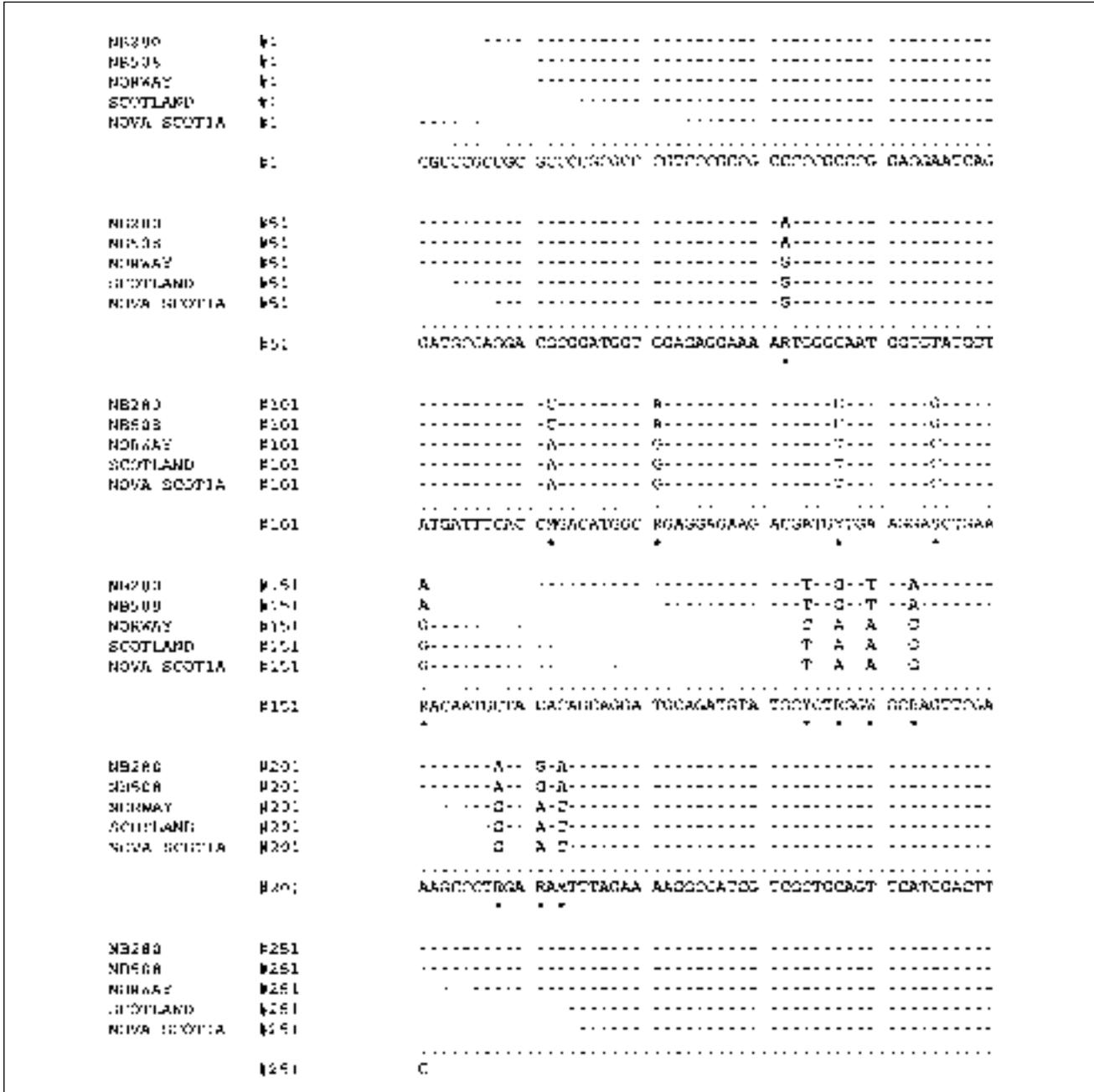


**Figure 1a**—Sequence analysis of RT-PCR products generated by segment 6 primers. Nucleotide 1–40: GC clamp; 1–63: HAFnew (GC clamp) primer; 257–277: 1414mod primer. The bottom line denotes the consensus sequence. Differences are marked by an asterisk (\*).



**Figure 1b**—Sequence analysis of RT-PCR products generated by segment 7 primers. Nucleotide 1–40: GC clamp; 1–62: MAF1 (GC clamp) primer; 432–454 MAR1 primer. The bottom line denotes the consensus sequence. Differences are marked by an asterisk (\*).





**Figure 1c**—Sequence analysis of RT-PCR products generated by segment 8 primers. Nucleotide 1–40: GC clamp; 1–62; NBFA3 (GC clamp) primer; 231–251: RA3 primer. The bottom line denotes the consensus sequence. Differences are marked by an asterisk (\*).

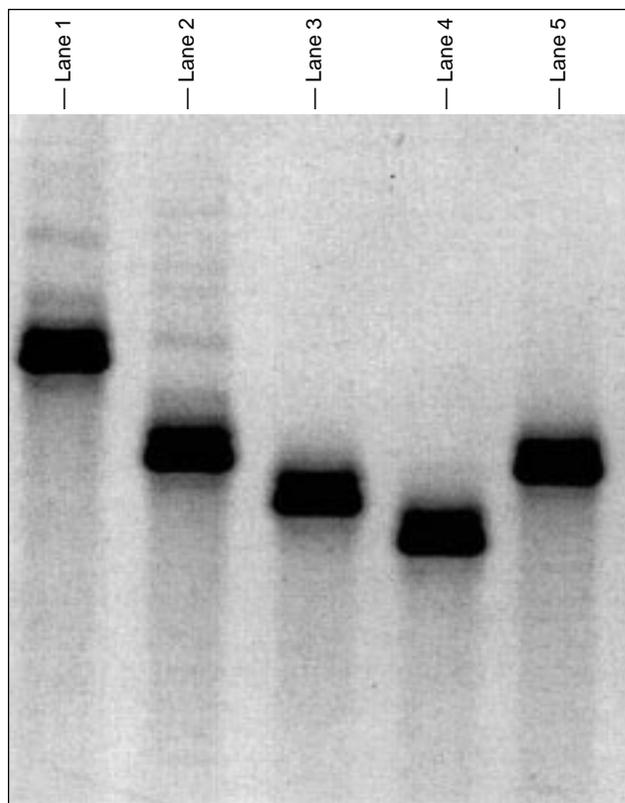
sequenced for comparison with DGGE data (fig. 1a–c).

To optimize DGGE conditions appropriate for the resolution of each RT–PCR product, initial electrophoresis conditions involved a broad gradient of denaturant (e.g., 20–80 percent or 30–80 percent urea–formamide mixture) in a 6-percent acrylamide gel run at 80 V for 17 h at a constant buffer temperature of 60 °C. By estimating the concentration of denaturant that terminated the migration of discrete RT–PCR products for each segment, subsequent conditions incorporated progressively narrower ranges of denaturant to provide optimal resolution of fragments. For the segment 6 and 8 RT–PCR products, a 45- to 65-percent gradient gel was selected for separation of fragments, but a 35- to 55-percent gradient gel was chosen for segment 7 products.

When RT–PCR/DGGE was run on segment 6 for the five reference isolates, five distinct bands were seen (fig. 2a). Different migration patterns correlated with nucleotide sequence differences (fig. 1a). The NB508 and Nova Scotian standards were only marginally resolved under these electrophoretic conditions. Accordingly, we made an effort to increase the resolution between these two isolates by decreasing the range of gradient to 10 percent (i.e., a 50- to 60-percent gradient gel) and by varying the electrophoretic time. Still, further improvement in resolution was not obtained. Nevertheless, the availability of well-resolved RT–PCR products from segments 7 and 8 easily differentiated these two isolates (see figs. 2b and c).

When the RT–PCR products that had the same migration pattern under non-denaturing conditions were run by DGGE for segment 7, four distinct migration patterns were observed (fig. 2b). The variation in migration could again be explained by nucleotide sequence variation (fig. 1b). Interestingly, isolates NB280 and NB508, which differed at two nucleotide positions (positions 110 and 146; fig. 1b) had seemingly identical migration patterns.

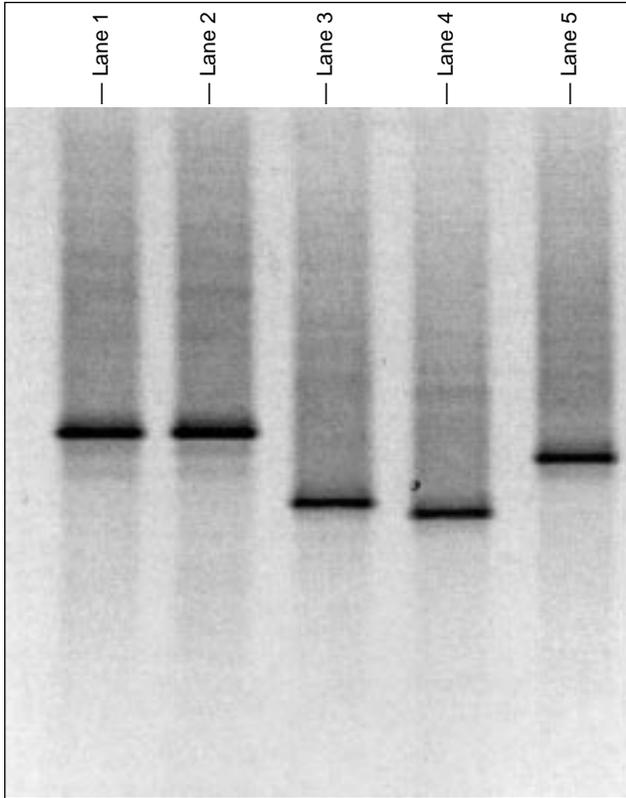
When the segment 8 RT–PCR products were run on DGGE (fig. 2c), three distinct migration



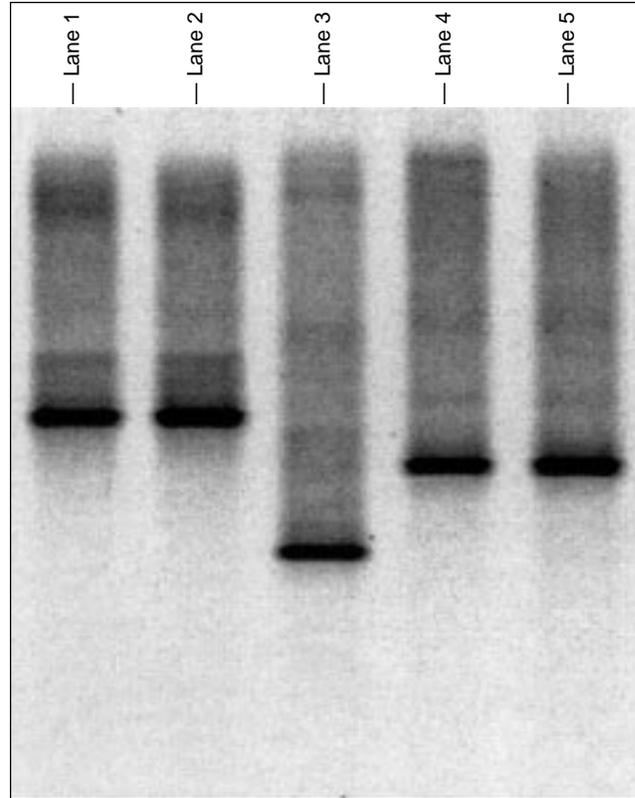
**Figure 2a**—DGGE analysis of segment 6 RT–PCR products for ISAv reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). RT–PCR products were electrophoresed in a 45- to 65-percent denaturant gel at 80 V, 17 h, 60 °C.

patterns were observed: NB280 and NB508 migrated the shortest distance, Scottish and Nova Scotian isolates migrated farther, and the Norwegian isolate displayed the farthest migration. Migration patterns correlated with nucleotide variation detected by sequencing (fig. 1c). The NB280 and NB508 isolates were identical in sequence as were the Scottish and Nova Scotian isolates. Interestingly, although the Norwegian isolate differed from the Scottish and Nova Scotian isolates by only one nucleotide (position 184; fig. 1c), the change was from T to C, and it had a noticeable effect on migration.

To further test the ability of this assay to type New Brunswick ISAv, we selected five isolates from different sites in the Bay of Fundy and ran them in the



**Figure 2b**—DGGE analysis of segment 7 RT-PCR products for ISAv reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). RT-PCR products were electrophoresed in a 35- to 55-percent denaturant gel at 80 V, 17 h, 60 °C.



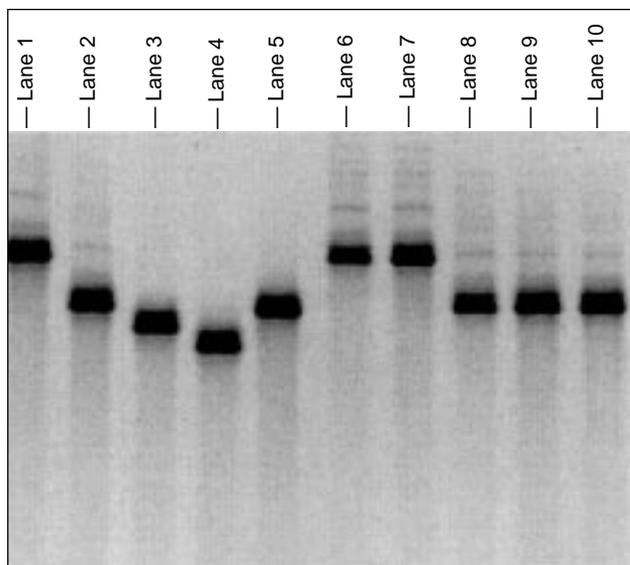
**Figure 2c**—DGGE analysis of segment 8 RT-PCR products for ISAv reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). RT-PCR products were electrophoresed in a 45- to 65-percent denaturant gel at 80 V, 17 h, 60 °C.

DGGE system against the five reference ISAv isolates for segments 6, 7, and 8 RT-PCR products. Segment 6 RT-PCR/DGGE analysis typed isolates NB877 and NB028 as reference standard NB280, whereas isolates NB049, NB002, and NB458 were typed as reference standard NB508 (fig. 3a). Sequencing analysis of NB877, NB028, NB049, NB002, and NB458 (data not shown) confirmed the identifications made by the RT-PCR/DGGE assay. RT-PCR and DGGE analysis of these isolates based on segment 7 sequences (fig. 3b) typed these as New Brunswick strains because migration patterns were equivalent to those for reference standards NB280 and NB508, as did the analysis based on segment 8 sequences (fig. 3c).

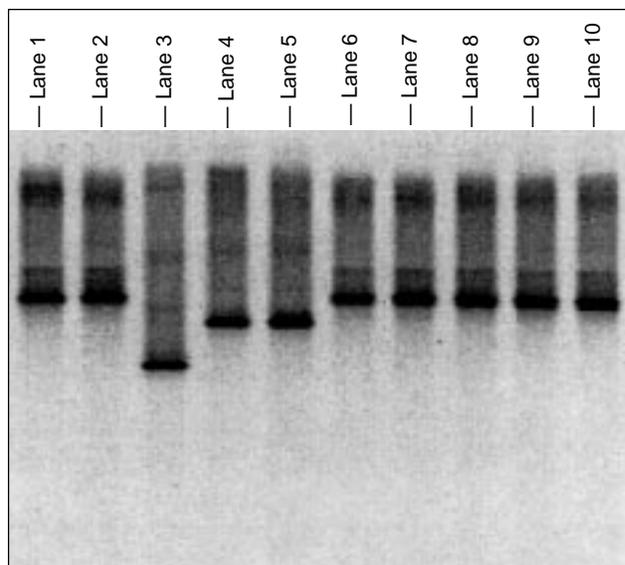
## Discussion

We have described three different RT-PCR/DGGE systems based on segment 6, 7, and 8 sequences. Due to the low genetic variability in segment 8 amongst ISAv isolates, the assay using segment 8 primers rapidly distinguished New Brunswick isolates from Nova Scotian and European isolates. The assay using segment 7 primers rapidly distinguished New Brunswick isolates from Nova Scotian, Norwegian, and Scottish isolates. The inability to distinguish the two New Brunswick reference isolates based on segment 7 sequences, despite the fact that they differed at two nucleotide positions (positions 110 and 146), is unusual since the substitution of

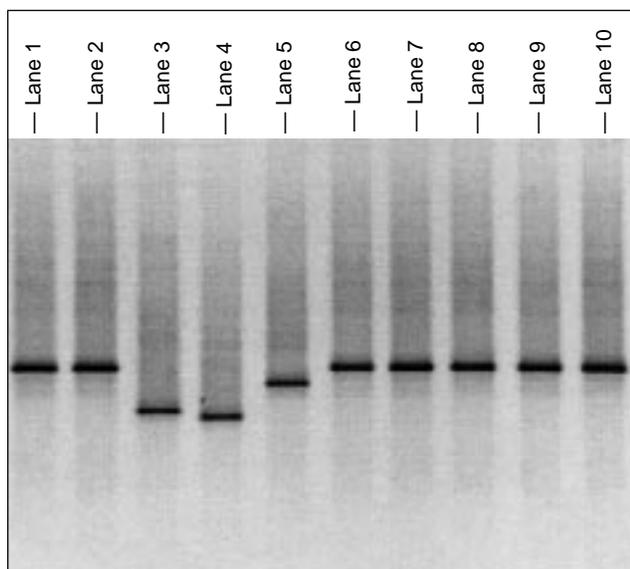
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**Figure 3a**—Typing of New Brunswick isolates using segment 6 RT-PCR/DGGE analysis. Reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). Test isolates: NB877 (lane 6), NB028 (lane 7), NB049 (lane 8), NB002 (lane 9), NB458 (lane 10). RT-PCR products were run in a 45- to 65-percent denaturant gel at 80 V, 17 h, 60 °C.



**Figure 3c**—Typing of New Brunswick isolates using segment 8 RT-PCR/DGGE analysis. Reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). Test isolates: NB877 (lane 6), NB028 (lane 7), NB049 (lane 8), NB002 (lane 9), NB458 (lane 10). RT-PCR products were run in a 45- to 65-percent denaturant gel at 80 V, 17 h, 60 °C.



**Figure 3b**—Typing of New Brunswick isolates using segment 7 RT-PCR/DGGE analysis. Reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). Test isolates: NB877 (lane 6), NB028 (lane 7), NB049 (lane 8), NB002 (lane 9), NB458 (lane 10). RT-PCR products were run in a 35- to 55-percent denaturant gel at 80 V, 17 h, 60 °C.

G (NB280) and A (NB508) would suggest that this was possible. If the problem lies with the proximity of these two base pairs to the GC clamp and their location in one of the higher melting domains, then movement of this clamp to the 3' primer might allow for the visualization of these differences. However, Winmelt analysis would suggest that movement of the GC clamp to the 3' primer may impede the detection of other nucleotide differences among ISAv isolates. Consequently, we are currently investigating other DGGE conditions to resolve this issue.

Using RT-PCR/DGGE analysis of segment 6, it was possible to differentiate all five ISAv reference isolates, corroborating previous suggestions that the variability in this segment may be enough to separate ISAv isolates (Krossøy et al. 2001). The contribution of more isolates for use as reference standards that are significantly different in the HPR region of segment 6 would increase the potential for typing and tracking ISAv isolates obtained in future screening programs. If differences in segment 7 in the New

Brunswick isolates can be differentiated, this segment may prove more appropriate to assay relatedness of strains as the differences here likely arise gradually by point mutations, not like the observed HPR variation, which likely occurs rapidly by deletion events. More informative than RFLP analysis and more rapid and inexpensive than sequencing, the DGGE analysis of RT-PCR products is a useful method to continuously monitor the types of ISAV that currently exist, to monitor their spread, and to identify the emergence of new strains.

## Acknowledgments

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# The Genetics of Infectious Salmon Anemia Virus

Carey O. Cunningham and Michael Snow<sup>1</sup>

**Abstract:** The complete genome sequence of infectious salmon anemia virus (ISAv) has been determined. Although ISAv is classified as an orthomyxovirus based on physical characteristics and genome type, it has a genome organization that is strikingly different from that found in influenza viruses. Thus it has not been possible to extrapolate directly from influenza to ISAv, and the genes encoding only one polymerase and the hemagglutinin protein have been established to date. Genetic variation can be

extremely useful in epizootiological studies, but increased knowledge of variation in genes such as the hemagglutinin has revealed that analysis of the variation can be problematic without detailed understanding of the mechanisms that create it. Detection of ISAv and diagnosis of ISA has come to rely heavily upon molecular tests such as the polymerase chain reaction (PCR). These are currently the most sensitive methods available and have proven reliable in routine use.

## Introduction

The discovery of the complete genome sequence of ISAv and analysis of the products encoded by these genes has progressed rapidly, indicating both the importance of the disease and the value of genetic investigations for aquaculture research and development. Although ISAv is an orthomyxovirus, it has a genome organization that is strikingly different from that found in influenza viruses. We will review the work of major groups of researchers to elucidate the genome of the virus and analyze individual genes. Detection of ISAv and diagnosis of ISA relies heavily on molecular tests such as the polymerase chain reaction (PCR). We will also discuss the available methods and the interpretation of results.

## Chronology of ISAv Gene Discovery

The genomic organization of ISAv was described by Mjaaland et al. (1997) as a genome typical of members of the *Orthomyxoviridae* family, being segmented, single-stranded, and negative sense. The total genome of approximately 14.5 kb was shown to be composed of eight segments ranging from 1 to 2.3 kb. The sequence of the smallest segment, 8, was obtained and used to design PCR primers. The sequence of segment 8 indicated the presence of two open reading frames (ORFs) which, like most ISAv segments, did not have significant homology with any other available nucleotide or amino acid sequence. Based on extrapolation from

the genome organization of the influenza viruses, it was widely assumed that this segment encoded the NS 1 and 2 proteins (Blake et al. 1999, Kibenge et al. 2000).

Krossøy et al. (1999) described the sequence from segment 2 of ISAv. This is the only gene in which significant homology with other members of the *Orthomyxoviridae* has been identified to date. The 2.3 kb segment encodes one protein with motifs that are conserved across the *Orthomyxoviridae* and in RNA-dependent RNA polymerase genes in general. Based on phylogenetic analysis using this sequence, Krossøy's work showed that ISAv is distantly related to the other *Orthomyxoviridae* and more closely related to the influenza viruses than the Thogoto viruses. Hence, Krossøy et al. proposed that a new genus be created and the name Isavirus has been proposed by the International Committee on Taxonomy of Viruses Index of Viruses, version 3 (<http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm>) for this genus, in preference to *Aquaorthomyxovirus*, which was suggested by Krossøy et al. (1999).

Krossøy et al. (1999) noted conserved regions of nucleotides at the 5' termini of ISAv cDNA sequences. Conservation of sequence at the 3' termini of ISAv segments 7 and 8, and complementarity with the conserved regions at the 5' termini were described in detail by Sandvik et al. (2000). These features have since been identified in all ISAv segments. Conservation and complementarity of termini sequences are characteristics of orthomyxoviruses that enable formation of panhandle structures that are important for viral RNA stability, and the terminal sequences are important to initiate transcription. The conservation of 5' and 3' sequence in ISAv, and evidence that ISAv requires capped

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cellular mRNA for replication, support inclusion of ISAv in the *Orthomyxoviridae*.

By 2001, groups working in Canada and Scotland had published further information on the genome of ISAv. Snow and Cunningham (2001) described segment 3 and analysis of the nucleotide, and predicted amino acid sequences suggested that that this segment encoded the nucleoprotein (NP). The predicted molecular weight of the gene product, 71 kDa, correlated well with previous estimates of structural proteins (Falk et al. 1997, Griffiths et al. 2001). Ritchie et al. (2001b) also described the sequence of segment 3 and included segment 4, possibly another polymerase-encoding gene. The work of the Canadian group showed that the putative NP was immunoreactive, a finding that may be particularly significant for development of vaccines against ISA.

Also important for vaccine development, and perhaps the most sought-after gene, was the hemagglutinin (HA). The sequence of this gene and direct demonstration that the gene product resulted in HA via transfected cells was reported by Norwegian and Scottish groups in 2001 (Krossøy et al. 2001a, Rimstad et al. 2001). The product of this gene was analyzed by Griffiths et al. in the same year. The HA gene product reacts with a monoclonal antibody developed some years previously by Falk et al. (1998). A striking feature of this gene is the highly polymorphic region (HPR) predicted to lie immediately outside the viral envelope.

All segments of the ISAv genome were presented in a single publication by Clouthier et al. (2002). A detailed analysis of segment 7 was presented by Biering et al. (2002) and Ritchie et al. (2002). Although segment 7 was initially thought to encode the matrix proteins, as it was widely believed that the NS genes were encoded by segment 8, Biering et al. (2002) presented evidence that segment 7 probably encodes the equivalent of the influenza nonstructural proteins while segment 8 encodes structural (possibly matrix) proteins.

Work continues to complete analysis of the protein encoded by segment 5. Thus, the entire

genome of ISAv and the identity of some gene products have been discovered. An overview of the genome of ISAv is presented in table 1.

## Comparison of ISAv and Other *Orthomyxoviridae*

During the work to determine the ISAv genome, it has been tempting to draw parallels with the other *Orthomyxoviridae*. The gene order of ISAv has important differences from that of influenza, and hindsight has shown that this extrapolation caused confusion, such as the suggestion that segment 7 may encode matrix proteins (Ritchie et al. 2002). The table comparing the genomes of the *Orthomyxoviridae* from Mjaaland et al. (1997) can now be extended to give a more complete picture of these differences (table 2).

The general composition of the ISAv genome resembles the other *Orthomyxoviridae* in that the eight segments appear to encode polymerase, nucleoprotein, HA, esterase, matrix, and nonstructural proteins. Whereas influenza A and B have HA and neuraminidase genes on different segments, influenza C carries HA, esterase, and fusion genes on a single segment. ISAv has esterase, not neuraminidase, activity, and it has been suggested that the esterase may be encoded on the same segment as the HA, segment 6 (Knut Falk, personal communication). The location of the gene conferring fusion activity has not been confirmed.

It is easy to envisage that genetic drift during the evolution of the *Orthomyxoviridae* could lead to a different gene order, giving rise to situations such as influenza A having a nucleoprotein segment significantly smaller than the polymerases whereas in ISAv the nucleoprotein is larger than one polymerase. However, the evolution of different arrangement of segments with multiple genes indicates that genetic rearrangement has also occurred on a much larger scale. Mechanisms of recombination and reassortment have been proposed for the *Orthomyxoviridae* (Gibbs et al. 2001, McCullers et al. 1999, Worobey and Holmes 1999); perhaps these have

been instrumental in bringing about the diversity of genome organisation within the genus.

Homologous recombination has been suggested as a possible mechanism for generating the large variation seen in the HA gene sequence of ISAv (Devold et al. 2001). Homologous recombination and reassortment can only occur when two different forms of virus occur in the same cell. Because more than one type of ISAv has been found in fish from the same salmon farm (Cunningham et al. 2002, Mjaaland et al. 2002), it is possible that reassortment could also occur in ISAv.

## Genetic Diversity of ISAv

Early comparisons of sequences of ISAv segments 2 and 8 showed large differences between isolates from Norway and Scotland and those from Canada (Blake et al. 1999, Cunningham and Snow 2000, Kibenge et al. 2000, Lovely et al. 1999, Ritchie et al. 2001a). The significant sequence variation between isolates provides evidence that ISAv has existed for a long time. It has been postulated that different ISAv isolates from Europe and Canada diverged approximately 100 years ago (Krossøy et al. 2001b).

**Table 1—Infectious salmon anemia virus (ISAv) genes and proteins described as of June 2002 and comparison of gene order with influenza C**

Gene	Segment ISAv	Segment 'flu C	Sequence	Segment (bp)	ORF (bp)	Protein (kDa)	Descriptions
Polymerase	1	1	AF404304 AJ514403	>1749 2205	>1749 2169	80	Clouthier et al. 2002 Snow et al. 2003
Polymerase	2	2	AJ002475 AF404346	2245 2185	2127 2127	80.5	Krossøy et al. 1999 Clouthier et al. 2002
Nucleoprotein (NP)	3	5	AJ276858 AF306549 AF404345	2069 2042 2046	1851 1851 1851	71 68 72	Snow and Cunningham 2001 Aspehaug et al. 2001 Ritchie et al. 2001 Clouthier et al. 2002
Polymerase	4	3	AF306548 AF404344	1805 1787	1737 1737	65.3	Ritchie et al. 2001 Clouthier et al. 2002
Unconfirmed	5			1560 1504		53 47	Snow, unpublished Aspehaug et al. 2001 Clouthier et al. 2002
Hemagglutinin/esterase (HE)	6	4 HEF	AF220607/ AJ276859 AF302799– AF302803 AF309075 AF404342	1326/1321 1053–1261 1323	1167/1175 >1053–1168 1185	43 38/72 42	Rimstad et al. 2001 Krossøy et al. 2001 Aspehaug et al. 2001 Griffiths et al. 2001 Clouthier et al. 2002
Nonstructural proteins (NS)	7	7	AF328627 AY044132 AF404341 AF429989–90	1159 1006 966	903/480 903/522 771/441	35.4/17.5	Ritchie et al. 2002 Biering et al. 2002 Clouthier et al. 2002
Matrix proteins (M)	8	6	Y10404 AF404340	930 736	779/>459 705/552	24	Mjaaland et al. 1997 Aspehaug et al. 2001, Biering et al. 2002 Clouthier et al. 2002

Note: Where > is shown in open reading frame (ORF) size, sequence is incomplete. Only polymerase (segment 2) and HA (segment 6) gene products have been confirmed in ISAv.

The presence of another variant of ISAv in Canada that has much greater sequence similarity to European than to the Canadian New Brunswick ISAv (Ritchie et al. 2001a and 2002) indicates that the distribution of ISAv types is not restricted geographically. The virus may be widely distributed throughout the Northern Hemisphere, and this distribution may be influenced by anthropogenic factors.

Once the HA gene of ISAv had been identified and analyzed, the HPR of the gene became the focus of much interest. The function of this HPR is unclear, but it has been used to differentiate ISAv strains (Devold et al. 2001, Kibenge et al. 2001a). Numerous distinct strains can be differentiated, based on the amino acid sequence within the HPR, and these HPR

types may provide useful epizootiological markers (Devold et al. 2001). However, there are isolates that have widely different HPR sequences but very similar sequence elsewhere in the gene and, conversely, isolates with the same or very similar HPR sequences but large differences elsewhere in this or other segments. These variabilities complicate the use of the HPR alone in epizootiology. Eighteen different HPR types have been identified so far (Cunningham et al. 2002, Mjaaland et al. 2002).

Functional differences in cytopathogenicity and infectivity or pathogenicity have been noted between ISAv isolates (Kibenge et al. 2000, Griffiths et al. 2001, Ritchie et al. 2001a), and antigenic variation may be related to sequence variation in the HPR (Kibenge et al. 2001a). Analysis of this HPR also

**Table 2—Comparison of genome structure within the *Orthomyxoviridae* showing size (in kb) and products of genome segments**

Genome segment	----- Influenza -----			Thogoto	Dhori	ISAv
	A	B	C			
1	2.34 PB2	2.38 PB1	2.35 P1	2.38 PB2	ND	2.20 PB2
2	2.34 PB1	2.38 PB2	2.35 P2	2.21 PB1	2.2 PB1	2.24 PB1
3	2.23 PA	2.30 PA	2.15 P3	1.92 PA	ND	2.07 NP
4	1.75 HA	1.88 HA	2.07 HEF	1.57 G	1.6 G	1.80 P
5	1.56 NP	1.84 NP	1.80 NP	1.41 NP	1.5 NP	1.56 ?
6	1.41 NA	1.40 NA NB	1.18 M	>0.96 M	0.96 M	1.32 HE
7	1.03 M	1.19 M	0.93 NS	—	ND	1.16 NS
8	0.89 NS	1.09 NS	—	—	—	0.93 M
Total	13.6	14.5	12.9	10.5	10	13.28

Note: Table based on table 2 in Mjaaland et al. (1997) with the addition of ISAv data and Thogoto virus data from European Molecular Biology Laboratory (EMBL) nucleotide database, June 2002.

**Gene products**

ND: No data available.

—: Segment not present.

P, P1, P2, P3, PB1, PB2, PA: Polymerase

HA: Hemagglutinin; HEF: Hemagglutinin/Esterase/Fusion

HE: Hemagglutinin/esterase; ?: Gene product unconfirmed.

NP: Nucleoprotein

NA, NB: Neuraminidase

M: Matrix

NS: Nonstructural

G: Glycoprotein

demonstrated the diversity of Canadian New Brunswick and many European ISAv isolates, but the addition of further sequences from Norway, some of which have the same HPR type as the New Brunswick ISAv (Krossøy et al. 2001a), has shown that the isolate may not be unique to Canada. Intuitively, it might be expected that an RNA virus that has been present for approximately 100 years and infects marine fish (including Atlantic salmon) might now exist as a pool of genetic variants that have evolved different phenotypes. Very likely, other variants will be identified in future.

It was proposed that the large variety of ISAv HA sequences could be produced by homologous recombination (Devold et al. 2001). Since that publication, the discovery of a novel HA sequence that contains all of the sequence motifs previously reported in one single sequence (Cunningham et al. 2002) suggests that differential deletion may be used, instead of, or as well as, homologous recombination, to generate antigenically or functionally diverse forms of ISAv HA.

The diversity of sequence in ISAv requires care in analysis. Where recombination or reassortment occur, more straightforward methods of phylogenetic analysis are inappropriate (Schierup and Hein 2000). Thus the HA gene, or the HPR, will require different methods of analysis than other genes.

## Molecular Epizootiology

The investigation of possible links between outbreaks of ISA has benefited from molecular study of the virus. Evidence of viral transmission was provided in the form of HA HPR sequences (Devold et al. 2001). In Scotland, identical sequences found from ISAv-infected fish in different farms provided evidence that the outbreak stemmed from a single point source (Stagg et al. 2001).

While the HPR may provide a good marker of different types or strains of ISAv, viral samples with the same HPR type might not necessarily be directly linked. Differential deletion might produce the same HPR type from viruses that have significant

divergence in other parts of the genome, and even in the same segment as the HPR. Therefore, the HPR alone is not necessarily a suitable marker for epizootiological studies. Nevertheless, the value of molecular data for this work is clear.

## Molecular Diagnosis of ISA

Molecular methods of detecting ISAv have assumed a leading role in the diagnosis and monitoring of this disease. Mjaaland et al. (1997) developed PCR primers that have proven reliable and sensitive for diagnostic purposes and are still in use on a large scale. Other primers developed from segment 8 sequences have been developed (Devold et al. 2000, Løvdal and Enger 2002), with those of Devold et al. employed extensively in Canada and Norway (Griffiths and Melville, 2000 Kibenge et al. 2001b). While primers for other segments of the genome have been developed, those that anneal to segment 8 seem to be most sensitive for routine diagnostic purposes (see Mjaaland et al 2002). As that segment appears to encode the matrix proteins (Biering et al. 2002), this sensitivity is to be expected, as transcripts from the equivalent segment are very abundant in influenza viruses. In addition to sensitivity, molecular detection offers advantages such as the ability to identify the tissue localization of the virus by *in situ* probe hybridization (Gregory 2002).

Kidney has been routinely used as the starting material for PCR tests. No significant difference has been found in PCR results from kidney, heart, gill, liver, and blood, so kidney is routinely sampled for ease of sampling. Nonlethal methods of sampling for PCR (Griffiths and Melville 2000) are extremely useful for broodfish or wild stocks.

Detection of ISAv by PCR is the most sensitive method of detection to date (Devold et al. 2000, Mjaaland et al. 2002, Opitz et al. 2000). The virus can be detected by PCR as early as 5 days postinfection (Mikalsen et al. 2001) within a variety of organs (Rimstad et al. 1999). The pattern of PCR positivity is followed approximately 10 days later by a parallel mortality pattern. However, it is likely that

variability in the susceptibilities of fish, husbandry practices, and environmental factors will significantly influence the outcome of infection with ISAv, and finding PCR-positive fish does not necessarily predict that mortality will follow.

The application of PCR detection methods for fish disease surveillance has attracted considerable debate regarding specificity and the likelihood of detecting nonviable pathogens. The specificity of ISAv detection can be confirmed by probe hybridization to PCR products (McBeath et al. 2000) or by sequencing the product. Amplification of parts of more than one segment of the genome is a particularly good indication of the presence of intact virus but may require higher levels of infection than detection of a small portion of segment 8 alone. Low levels of virus may be detected after concentration or antibody capture, and work continues to develop additional antibodies for this purpose, using phage display as well as immunization with recombinant protein, synthetic peptides and DNA vaccine vectors.

## Conclusions

The investigation of the genetics of ISAv has yielded an enormous amount of extremely valuable information for identification and analysis of ISA and has also provided data of great scientific interest. Molecular methodology is an integral part of diagnosis of ISAv and will continue to provide more tools and information to improve detection of this virus. Epizootiological studies have benefited from ISAv sequence data, and development of vaccines may proceed more rapidly through application of recombinant or DNA vaccines. Analysis of the *Orthomyxoviridae* has been given a new twist with the discovery and examination of this distant relative of the influenza viruses, and models of the evolution of the *Orthomyxoviridae* and ISAv in the future should prove fascinating.

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# Infectious Salmon Anemia in Norway and the Faroe Islands: An Industrial Approach

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**Abstract:** After existing as a spot-vice disease during most of the 1990s, infectious salmon anemia (ISA) emerged as a severe threat to the salmon farming industry throughout Norway in 2001. Simultaneously, the disease also developed with increasing severity in the Faroe Islands. Records comparing the number of outbreaks today with historic data will be shown both for Norway and the Faroe Islands. Although ISA has consistently caused great losses at an affected site, its economic importance has dramatically changed recently. Insurance indemnification programs effectively reduced the economic losses for the scarce

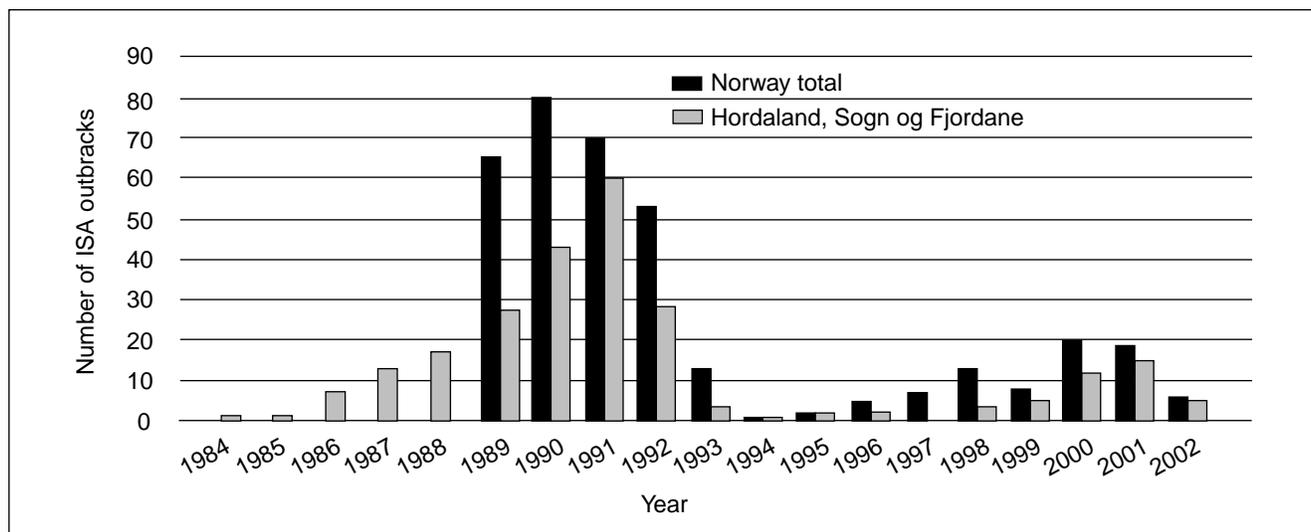
number of outbreaks that occurred during the 1990s, but the present increase of ISA outbreaks has reduced the willingness of insurance companies to provide sustained coverage. This situation has forced the industry and governmental agencies to establish effective measures against the disease. Figures demonstrating the economic impact of ISA to the industry will be presented. This paper will further discuss how changes made in the salmon industry during the last decade have facilitated the reemergence of a low-virulence agent like ISAv.

## Disease Status

During most of the 1990s, infectious salmon anemia (ISA) was classified as a spot-vice disease in Norway (Vågsholm et al. 1994) but reemerged as a severe threat to the Atlantic salmon (*Salmo salar*) industry in 2001 (Martin Binde, personal communication). Simultaneous with its reemergence in Norwegian aquaculture (fig. 1), ISA also developed with increasing severity in the Faroe Islands (Peter Østergaard, personal communication). When ISA is diagnosed in Norway, governmental authorities

subject the affected site to specific restrictions. If any fish on the farm contract this disease, the entire population is affected by the restrictions.

The development of ISA in Norway occurred predominantly within the counties of Hordaland and Sogn og Fjordane, which represent 54 percent of the total number of outbreaks throughout the period (Eide 1992; Martin Binde, personal communication). From 1997 until 2002, these counties reported 56 percent of the total number of Norwegian outbreaks. Compared with the percentage of production, which is 29, this area is overrepresented



**Figure 1**—Number of sites subject to restrictions due to ISA diagnosis in Norway totally and in the counties of Hordaland and Sogn og Fjordane.

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in the incidence of ISA (Martin Binde, personal communication). The record of ISA in the Faroe Islands is more recent and not as historically established as it is in Norway (Peter Østergaard, personal communication).

## **Effect on Industry**

Once an outbreak of ISA occurs, current management practices require immediate harvest or culling of the affected fish. Similarly, other groups of salmon within the affected site are subject to accelerated harvest if their mortality exceeds 0.5 per thousand fish per day per pen. If mortality is lower than this limit, production may proceed as normal, and harvest is subsequently prolonged. In other areas of the affected farm, one may agree on a harvesting plan to be instituted based on the severity of infection. This has been, and will remain, the legislative practice until a new national contingency plan is implemented in Norway. Healthy fish within the affected farm may be produced for human consumption without special trade restrictions.<sup>2</sup>

When outbreaks of ISA reach the levels seen through 2000 and 2001, the disease not only affects individual sites but also causes further complications for neighboring farms within the affected area. An epidemiologic study conducted in 1995 documented that the risk of ISA disease increased significantly if an adjacent site became infected (Jarp and Karlsen 1997).

Contagion of sites in this manner demonstrated the importance of site segregation because the risk of disease was considerably reduced if the distance between sites exceeded 5 km (Jarp and Karlsen 1997). This scenario was clearly evident in Gulen and Nordfjord, both of which are located in county Sogn og Fjordane, where the County Veterinary Office ordered a regional fallowing policy among affected farms. Obviously, this regime altered production plans for the farming companies involved,

and production was lost among those companies that did not have access to alternative sites.

## **ISA Insurance Programs**

Although outbreaks of ISA always cause loss of profit, the economic significance of the disease has varied. When insurance indemnification programs were introduced in 1996, limits were established for potential losses that made it possible for a farmer to survive even if the disease forced total depopulation of saleable fish. In some cases, the farmer could even recover the book value of the fish.

Insurance coverage for ISA indemnification has changed, however, now that the disease is more prevalent and more sites are affected. Naturally, indemnification against ISA losses has become less attractive to insurance companies. In certain regions, companies no longer allow fish farms to apply for ISA insurance; in other areas, insurance may be obtained only with an elevated owner risk. Unlike Norway, ISA insurance has never been commonly available in the Faroe Islands. Insurance with 50-percent owner risk was offered a short period before the ISA epidemic began. Presently [April 2003], insurance is not available.

The following profit-to-risk example is used to demonstrate how ISA became an intolerable financial risk for the industry. In this example, a site is selected where the fish weigh an average of 1 kg. This is the point in production schedules at which the effects of an ISA diagnosis hurt the most because it is at this weight that a fish has hardly any market value: it is too small to attract a fair price and it is very costly to slaughter. Consequently, fish less than 1 kg will usually be culled and used as a protein and fat resource in feed for fur-bearing animals. This utilization pattern produces financial loss. In fact, the farmer pays to dispose of the fish. All other scenarios involve less expense to the farmer because the fish either have a lower book value or there may be partial returns by selling some portion of the production lot in the market. Thus, the economic impact of ISA as a threat to the industry is best

<sup>2</sup>The new contingency plan will come into force from February 1, 2003.

visualized in a profit-to-risk ratio. This ratio expresses the relationship between predicted return on a three-concession site (meaning triplicate production schedules on each site per grown year class) divided by the estimated financial loss if ISA occurs. If such a ratio is 1, the risk equals the potential profit; thus one ISA outbreak is repaid by one site with normal production.

During 2000, the predicted profit per kilogram of fish harvested was very high because supply of farmed salmon in the market was lower than the commercial demand and the industry took advantage of cost-reducing factors in production. Accordingly, the declared insurance value often exceeded book value, and the market price also exceeded the insurance value. Therefore, insurance payments, in some cases, covered for book value even given 30-percent owner's risk. Practically, a maximum loss of \$140,000 could result if ISA occurred. [All cost figures are expressed in U.S. dollars.] Because other sites within the same company may produce normally and gained profit up to \$2.6 million, this risk was indeed tolerable. Thus, the profit-to-risk ratio was favorable to fish farming in 2000, but this trend did not persist (Ragnar Nystøyl, personal communication). Market prices affected the willingness of a farmer to grow more fish, which ultimately led to overproduction. During 2001, therefore, market prices dropped drastically. Meanwhile, the risk of having ISA contaminate a farm went up. This change resulted in an increased percentage of owner risk. Simultaneously, costs of production also increased because feed prices rose 25 cents/kg (internal data). Feed represents about half the cost of production. Consequently, the difference between insurance and book values zeroed or turned negative. Owing to the higher prevalence of ISA, owner risk went from 20 percent to 50 percent and, in some cases, even reached 100 percent. Predictable loss, if ISA occurred, consequently increased from \$128,000 in 2000 to between \$940,000 and \$1,740,000 in 2002. Within 2 years, the profit-to-risk ratio had changed to an

extremely nonfavorable status and turned financial realities (table 1) upside down for many farmers (Ragnar Nystøyl, personal communication). A profit-to-risk ratio of 0.02 means that 50 normal productions are required to repay 1 ISA outbreak.

Governmental agencies within the Faroe Islands treated ISA almost alike, but a distinct difference between the Faroes and Norway was that accessible sites were limited in the Faroes. Therefore, the number of fish per site in the Faroe Islands was even higher than in Norway. Numbers less than 1 million fish were rare and often there were as many 2 to 3 million fish produced per site. Unfortunately, production statistics were not available.

To date, production sites in the Faroe Islands have not sustained an escalating ISA mortality, and, therefore, production has not been disrupted totally. Losses have been limited to the actual pen culled or harvested. However, with prolonged culling of potentially diseased fish from a site, the worst-case scenario might develop in a matter of time. In such a situation the loss might go as high as \$3.8 million. Table 2 presents a scenario for a Faroe Island site, provided that all fish had to be culled at 1 kg during the 2002 production cycle (Ragnar Nystøyl and Peter Østergaard, personal communication).

The salmon farming industry generally depends on financial support to produce fish, and financial lending institutions are obviously risk oriented. Because of the profit-to-risk ratios presented previously, it became imperative for the industry in Norway and the Faroe Islands to improve the ISA situation. Together with governmental agencies, working groups were established to develop new national contingency plans. Except for local and geographic adaptations, both national plans developed similar principles to control and manage ISA by modernization of hygienic measures and implementation of vaccination programs.<sup>3</sup>

<sup>3</sup>As to the use of vaccines against ISA, this part of the national plans is still subject to EU/European Fair Trade Association approval.

**International Response to Infectious Salmon Anemia:  
Prevention, Control, and Eradication**

**Table 1—Infectious salmon anemia profit-to-risk ratio and potential loss for a Norwegian three-concession site holding 550,000 fish**

**a. Figures calculated at 20-percent owner risk for the year 2000.**

	Per kg (fish)	Total value per site <sup>1</sup>
Book value per kg	12.77	1,523,500
Insurance value per kg	13.17	1,743,500
Deductible % owner risk	20	348,700
Payable from insurance		1,394,800
Percent coverage		91.6
<b>Total loss per site</b>		<b>128,700</b>
Normal harvest size (guttet)	4.5	
Normal predicted profit per kg	11.06	
Normal predicted profit per site		2,623,500
<b>Profit-to-risk ratio</b>		<b>20.38</b>

**b. Figures calculated at 50-percent owner risk for the year 2002.**

	Per kg (fish)	Total value per site
Book value per kg	13.03	1,666,500
Insurance value per kg	12.64	1,452,000
Deductible % owner risk	50	726,000
Payable from insurance		726,000
Percent coverage		43.6
<b>Total loss per site</b>		<b>940,500</b>
Normal harvest size (guttet)	4.5	
Normal predicted profit per kg	10.013	
Normal predicted profit per site		32,175
<b>Profit-to-risk ratio</b>		<b>0.03</b>

**c. Figures calculated at 100-percent owner risk (no insurance coverage) for the year 2002.**

	Per kg (fish)	Total value per site
Book value per kg	13.17	1,743,500
Insurance value per kg	0	0
Deductible % owner risk	100	0
Payable from insurance		0
Percent coverage		0.0
<b>Total loss per site</b>		<b>1,743,500</b>
Normal harvest size (guttet)	4.5	
Normal predicted profit per kg	10.013	
Normal predicted profit per site		70,200
<b>Profit-to-risk ratio</b>		<b>0.02</b>

<sup>1</sup> Figures represent U.S. dollars at the exchange rate of \$1 = 7.58 Norwegian kroner.

**Table 2—Infectious salmon anaemia profit-to-risk ratio and potential loss for a typical site in the Faroe Islands holding 1.2 million fish during 2002: no insurance (100-percent owner risk)**

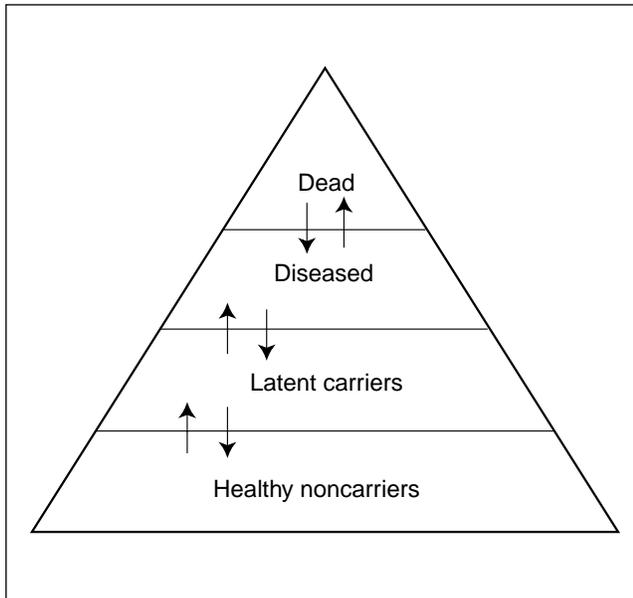
	Per kg (fish)	Total value per site <sup>1</sup>
Book value per kg	13.17	3,804,000
Insurance value per kg	0	0
Deductible % owner risk	100	0
Payable from insurance		0
Percent coverage		0.0
<b>Total loss per site</b>		<b>3,804,000</b>
Normal harvest size (guttet)	4.5	
Normal predicted profit per kg	10.013	
Normal predicted profit per site		70,200
<b>Profit-to-risk ratio</b>		<b>0.02</b>

<sup>1</sup> Figures represent U.S. dollars at the exchange rate of \$1 = 7.58 Norwegian kroner.

## Viral Infection of Atlantic Salmon

Even though ISA virus (ISAv) expresses different levels of virulence in the field, in vivo challenge trials and epidemiologic studies indicate that the virus is only moderately contagious. It is found in several fish species, including Atlantic salmon (*Salmo salar*) (Nylund et al. 1995), rainbow trout (*Oncorhynchus mykiss*) (Nylund et al. 1997), brown trout (*Salmo trutta*) (Nylund et al. 1994), Arctic charr (*Salvelinus alpinus*) (Snow et al. 2001), and herring (*Clupea harengus*) (Are Nylund, personal communication). Although this disease was not described before the advent of commercial Atlantic salmon aquaculture, it has been documented that the virus was present before it was first detected as the causative agent of ISA (Krossøy et al. 2001). The virus has evidently survived and propagated in wild fish. Unfortunately, we do not know much about viral replication and survival in wild populations. Such investigations would provide knowledge essential to combat the disease.

Evans and Brackman (1991) have presented a model (fig. 2) that depicts the presence of a pathogen



**Figure 2**—An iceberg model of infection depicting various infectious stages within a population as described by Evans and Brackman (1991).

in a host population. This model defines four different categories in which the host animal may find itself: (a) a healthy organism that does not harbor the pathogen, (b) a healthy organism that harbors a latent or carrier infection, (c) a carrier organism with clinical signs of disease, and (d) a dead organism that sheds the virus.

The ratio of individuals belonging to each category depends on environmental parameters, the virulence of the pathogen, and the susceptibility of the host. This model visualizes some of the problems associated with a moderately virulent disease like ISA. Strategy is, therefore, directed at management of the disease rather than elimination of the virus. Nevertheless, it is difficult to judge whether or not these measures are effective in a long-term perspective by assessing the outcome of clinical outbreaks alone. As long as knowledge of the underlying carrier status is scarce, epidemiologic control and transmission of the pathogen remain unclear. Analytical techniques applicable for such analyses have become available in the past 2 years, but methodologies still require validation.

Balances and imbalances prevail between different health categories in the pyramid (fig. 2) that may either manifest or suppress the expression of clinical disease and mortality. The balance may be altered owing to a variety of external and internal factors generally referred to as stress-inducing parameters. Normal production cycles contain several stressors, including operations (e.g., transport, sea-lice treatments, grading, and trenching), the maturation process itself, and the presence of concomitant diseases, among others. Also, host susceptibility may vary within salmon populations and among different strains.

Detection of ISAv with the reverse-transcriptase–polymerase chain reaction (RT–PCR) technique has been used since 2001 (Devold et al. 2000, Griffiths and Melville 2000). Although results from different laboratories may not be comparable, analyses have revealed that a wide range of carrier status exists among farmed salmon populations. Some farm sites may have a prevalence of latent carriers as high as 60 percent without manifestation of clinical ISA (internal data). Historically, it has been theorized that the vast majority of challenge originates with the shedding of viral particles from diseased and dead fish. Certainly, diseased and dead fish represent an important reservoir of infection. However, the virus must depend on other mechanisms of infection within the feral environment than those that facilitate transmission of the pathogen under conditions of intensive pen culture. Within the feral environment, infection may be affected by transmission of the virus across individuals within a shoal or when proximity of fish-to-fish contacts increases at the time of spawning. Dead or diseased fish are left behind and are not able to follow the shoal. Several viruses are present in gonadal fluids. The spawning pattern for anadromous or pelagic species presents three important factors for successful viral reinfection: (1) concentration of hosts, (2) exposure to contaminated gonadal fluids, and (3) the possibility of ingesting contaminated fertilized eggs or infected offspring. The identification of latent ISAv carriers in farmed salmon populations raises questions about whether the virus may have a

natural mechanism of spreading without producing clinical disease. This is an issue that can only be addressed by further research.

## Structural Changes That Affect Prevalence

During the 1988–92 epidemic of furunculosis, a thorough restructuring took place within the salmon-farming industry. Within this same period, ISA emerged and served as an additional stimulus for the restructuring process. In 1991, the first efficacious oil-adjuvant vaccine against furunculosis became commercially available, but neither pharmaceutical treatments nor vaccines were available to control ISA. Thus, control could only be effected by improving conventional hygienic measures:

- Separation of generations with “all in–all out” production
- Segregation of production sites by 3 km
- Daily collection of dead fish with dead-fish brills in each pen. Hygienic measures including the installation of silage tanks to handle dead fish at each site
- Abandonment of shallow and low-current sites with a preference for more exposed sites with good water exchange
- Fallowing of culture sites between generations
- Prohibition on movements of fish from site to site in the sea unless such movements are for the purpose of slaughter
- Implementation of sanitary packing and processing procedures by disinfection of effluent water
- Sanitation of wellboats between transports
- Ultraviolet disinfection of seawater intake in smolt farms

Collectively, these measures established new standards for the industry and helped to overcome its first epidemic of ISA. The increased reemergence of ISA since 2000, however, indicates that these measures were either insufficient or that personnel may have become somewhat lax in their attention to

detail. In the Faroe Islands, some of these fundamental hygienic measures have not yet been fully implemented (Peter Østergaard, personal communication).

In addition to the development of hygienic standards, one of the major changes in the industry during the last decade was a relocalization into new production sites. More exposed sites required better construction and sustained greater levels of production. Several concessions were also incorporated within the same site with the introduction of feed barges and automatic feed supplies. With 3 or 4 concessions on the same site, the number of fish increased to 550,000 to 680,000 (Norwegian Export Council 2002, Directorate of Fisheries 2002). This enhanced greater possibilities for ISA infection as follows.

The ratio between wild and farmed fish has changed considerably since 1990. Sea trout populations exhibit a regional life cycle, and the majority of the population does not move more than a few kilometers from their home river (Atle Kambestad, personal communication). In Norway, these populations are known to carry ISAv (Rolland and Nylund 1999, Nylund and Jakobsen 1995). Given that the size of the sea trout population remains rather stable on a regional basis, the ratio between sea trout and farmed salmon in some of the core areas of ISA had changed from about 1:15 in 1990 to 1:50 by summer 2002. In 1980, the ratio was approximately 1:1 (Atle Kambestad, personal communication). This affects whether or not we should consider wild or farmed fish as the main reservoir for the agent. In terms of population numbers, farmed fish may represent an independent reservoir.

The production pattern of transferring more fish per site also called for more fish per pen. In 1990, it was common to split a one-concession transfer into a minimum of three pens due to different sizes at the time of transfer. Today all fish may be put in one 90-m polar circle. Because smolts transferred per concession are now 2.5 times greater than they were in the past, present practices do indeed challenge the

smolt producer. It is biologically and technically difficult to produce 150,000 fish of consistent quality throughout the group. Practicalities often necessitate that the same pen contain smolts originating from different tanks in the smolt farm of different sizes and of inhomogeneous quality. This situation predisposes salmon in a communal pen to social turbulences and stressors that can trigger overt disease even if the number of latent carriers was originally low.

Also, the increased number of fish per site can enhance the infectious load in the adjacent area if ISA infection is established in the population. Even though ISA has reemerged during 2001, the number of affected sites still remains lower than peak numbers of sites reached in the early 1990s. This might suggest that the present situation is not as bad as it was 10 years ago. That inference, however, is slightly incorrect because it does not take into account that the number of fish per site has increased eightfold during the decade. In 1990, a site held an average of 60,000 to 70,000 fish. In 2001, a typical site was stocked with 550,000 to 680,000 fish (Norwegian Export Council 2002, Directorate of Fishery 2002). Again, reverting to the iceberg model, we find that the relative proportions of individuals belonging to each level are not necessarily changed but the numbers per category are much higher. Thus, if an ISA outbreak takes place, the number of fish shedding virus and consequently the total infectious load to the environment and adjacent sites are much higher. One may perceive that better sites and hygienic measures implemented by the industry would outweigh this. In fact, most of the industry has carried out higher stocking densities successfully. Still, the disease has been able to recur in some regions in Norway, such as Gulen and Nordfjord, which are core areas for the disease. Although the epidemiologic analysis conducted in 1995 has not been repeated, the disease pattern in 2000–02 does underline the same risk factors despite the good implementation of hygienic operational measures.

Another problem arising from high-stocked sites is the time required to empty the site after ISA

diagnosis has been verified. In general, emptying the site as soon as practically possible is perceived as the most essential part of the Norwegian Contingency Plan. Keeping the fish onsite while disease and infectious load are allowed to build up truly has a very negative impact on the adjacent sites as well as the environment. Still, harvesting capacity may be a limiting factor in many areas. In Norway, a fully stocked site of harvest-size fish may require as much as 50 working days to empty even with continuous harvest. In the Faroes, the job may take 100–120 working days.

Historically, it has been found that challenge has been brought in from affected sites or wild fish to nonaffected sites in various ways. This is still of vital importance. However, at a site holding 600,000 fish with a carrier status as high as 60 percent (internal results), chances may be higher that the agent originates from internal carriers (or diseased fish) than from outside introduction. A self-sustaining infection cycle cannot be excluded.

On the assumption that the virus is naturally persistent in wild fish, introduction of fish farming indeed established a new imbalance by introducing a relatively high number of susceptible hosts. The presence of serotypes in ISA<sub>v</sub> has never been documented or investigated. Variation in clinical manifestation is observed in the field. Whereas some sites experience very low mortality, others may observe an aggressive development of the disease with escalating mortality. However, the variation in mortality may also be explained by the developmental stage at which the disease is diagnosed on a farm. On the basis of evolutionary principles, one should expect that introduction of a high number of susceptible hosts would destabilize the existing natural balance between virus and hosts. Consequently, it may be questioned if a highly virulent virus has a greater ability to survive over time and spread across farming units (Are Nylund, personal communication). A comparative study of virulence across isolates from wild fish and farmed fish would verify this hypothesis.

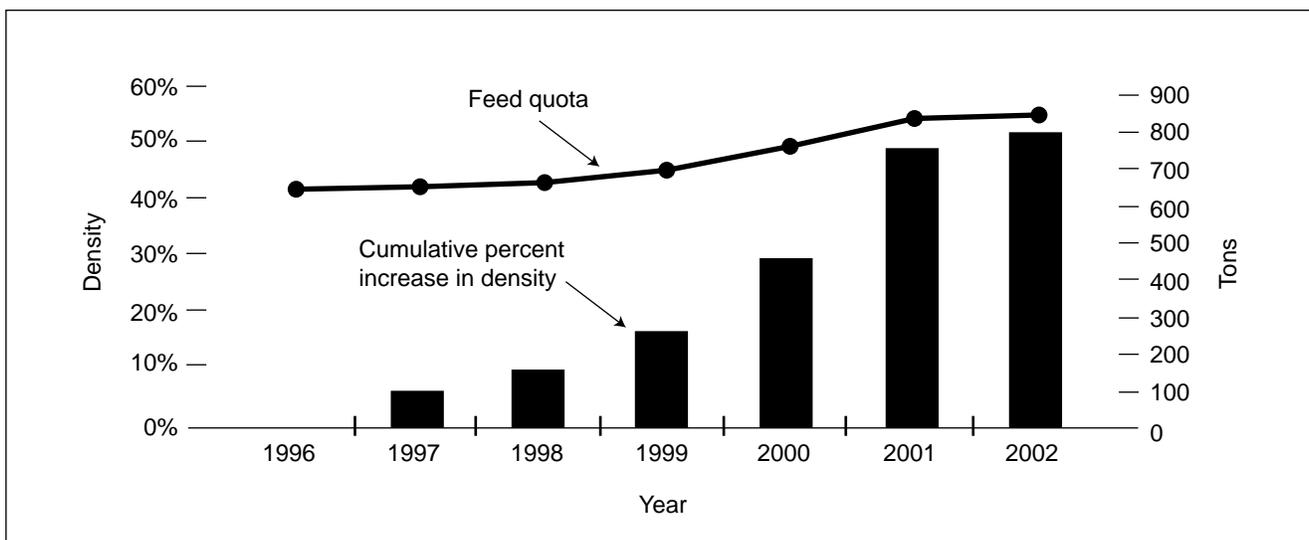
## Regulation of Production by Feed Quotas

In Norway, one production concession is based on a calculated volume, which is equivalent to 12,000 m<sup>3</sup>. Within a concession, there is a legislated density limit of 65 kg of fish per cubic meter of water and a guideline of 25 kg/m<sup>3</sup> based on health and animal welfare recommendations. Feed quotas per concession were instituted in 1996 within the parameters of The Salmon Agreement negotiated with the European Union to regulate supply of marketable salmon. The feed quotas have since been adjusted annually by the Norwegian Department of Fisheries. In 1996, the quota was 619 tons of feed, and it reached 847 tons in 2002 (fig. 3). This increase occurred without adjustment of the allowed concession volume, i.e., one concession could produce almost 50 percent more fish today than it did in 1996. Calculated maximum density has, therefore, increased by 50 percent. Although the farmers have moved towards bigger pens and are thus partly compensated by retrieving a bigger actual volume, the feed quota policy has not been assessed

from the perspective of salmon health. The production system, which is based on volume (cubic meters) is presently under revision.

To the extent that limitation of density is believed to reduce horizontal interaction between fish and limit the potential for contagion, measurements of density as kilograms of biomass per cubic meter of water are somewhat inappropriate. Infectious agents, whatever their nature, do not infect biomass per some arbitrary unit of volume: pathogens invade and infect individual hosts. Total biomass at a given site would have some relevance as a more appropriate measure than kilograms per cubic meter. Although density expressed as kilograms per cubic meter peaks just before grading and harvest, animal density (fish/cubic meter) peaks at transfer and declines throughout the production cycle (fig. 4). From a microbial perspective, the chances for infecting a new host are greatest at the start of the growth period. In figure 4, animal densities are calculated on the basis of a one-pen scenario.

Also relevant from a health point of view is examination of densities on a site basis. The volume defined by the whole farm as a unit will then apply.

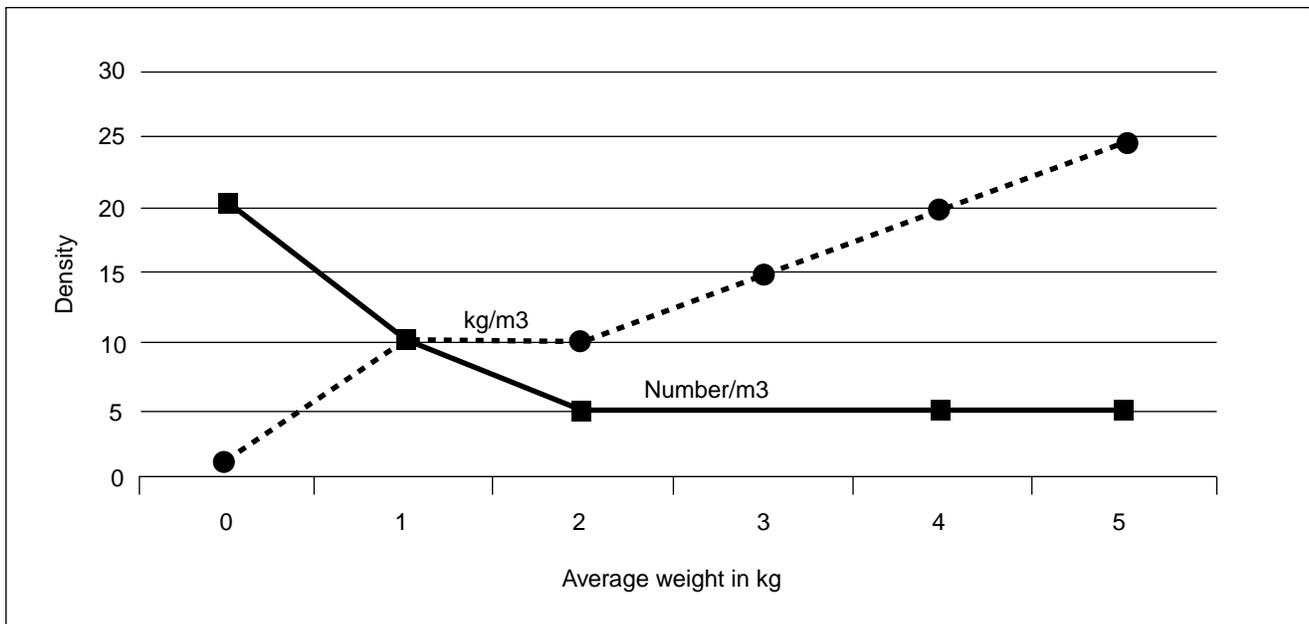


**Figure 3**—Percentage increase in density of cultured Norwegian Atlantic salmon based on increase of feed quotas from 1996 to 2002.

A site may consist of 6 to 12 pens. These are organized in a variety of ways in accordance with the type of farming construction used, anchoring possibilities, and the exposure of the site. Basically, two types are available—steel construction framing all pens in a rigid structure or flexible plastic units organized as separated pens. In the case of steel construction, the pens and consequently the fish, are positioned next to one another only with a walkway in between. With flexible plastic units, the units are spaced 10–40 m apart. In the latter case, the number of animals is stocked in a manner providing from two to six times more volume. Normally, the site is stocked according to pen volume, and that has to be taken into account. Nevertheless, the steel construction provides neither the animal density nor the biomass density afforded by the plastic circles based on site volume calculation (internal data; Tim O’Hara, personal communication).

Introduction of feed quotas also had dramatic consequences for feed composition. The amount of feed became the legislatively regulated limiting factor for how many tons of fish could be legally produced

per license. Certainly, in order to enhance production further, it was necessary to improve diets by increasing energy units per kilogram of feed. Since 1996, the fat content in salmon diets has increased from 27 to 41 percent, and the energy content has also increased accordingly. To increase the proportion of one ingredient in the feed, another has to be reduced. The only way of compensating is to increase the quality and specificity of the components being reduced. Simultaneously, high-energy feed allowed faster growth but reduced actual feed conversion rates (FCRs). Since 1996 and through 2001, the average FCR for the Norwegian salmon industry dropped from 1.216 to 1.175 (Ragnar Nystøyl, personal communication). As a consequence of this development, the prevalence of diseases during production had a tendency to increase. Increased growth capacity also requires a more concentrated feed for all other ingredients. Regrettably, science takes time to catch up with market requirements. In the meantime, the fish may become compromised in diet and be adversely affected immunologically.



**Figure 4**—Development of biomass density compared with animal density during growth in a standard salmon farm.

Fish transportation has been restructured along with the development of the industry. Some of these changes have not been favorable for preventive health. Expansion of carrying capacity by larger boats had a very positive impact on the cost of transport per kilogram of fish. However, densities during transport are greater than in the pen. Such transport also involves stress, and the fish are likely to receive and release viral agents en route to packing stations. If the fish originate from a site not subject to restrictions, full water exchange during transportation is allowed. If transportation routes pass other culture sites, this practice may enhance the spread of ISA (Murray et al. 2002). All transportation of live fish should be accomplished without any emission of infective agents. Boats should be self-contained, and smolt transportation should be carried out with particular boats periodically dedicated to this task.

Once salmon are transported to the packing facilities, they are often loaded into resting pens. These pens were established to decrease the time required to unload the wellboat and allow the fish to recover from transport stress. In reality, however, such a practice allows posttransport mortality to occur in an open environment with potential release of pathogens from the resting pens. Such facilities, therefore, should be brought onshore in closed compartments, where they will not become factors that can amplify pathogens and enhance disease processes in natural environments. When farming companies integrated and grew bigger, cost reductions were effected by replacing small and ineffective packing stations with new and larger facilities. To fill packing capacity, fish were transported across larger distances, which could result in pathogens such as the ISA virus being spread across broader areas.

Oftentimes, the interests of cost-effectiveness are at odds with biology, and this is also true with the transportation sector. Problems have been caused by trenching of fish in the pen before loading, the speed of unloading, the lack of rest for transport staffs, errors in hygienic and management routines,

and so forth. All these factors adversely affect the animal, the economic bottom line, and ultimately total environmental health. It is, therefore, a paradox that the industry itself has reduced transport cost to the point that spread of ISA has actually been enhanced (Murray et al. 2002).

Transportation of mixed species is practiced by some of the wellboat companies. Farmed fish and trimming (byproducts) from herring and mackerel are never transported simultaneously, but these products may be handled by the same boat on alternate trips. ISAv is isolated from, and verified capable of propagation in, herring (Are Nylund, personal communication). Byproducts consisting of intestines, stomachs, and livers definitely contain numerous viral particles. Additionally, this material is often chilled and stored from 1 to 3 days before transportation. Although the wellboat is washed with strong detergent and disinfectants before the following transport, the fat content of this material strongly adheres to most surfaces. Thus, complete disinfection of wellboat facilities after transport of such materials is a technical challenge. Satisfactory cleaning after transportation of byproducts and trimmings is virtually impossible. It is not hard to imagine what might happen if the next mission of a contaminated boat involved the movement of susceptible salmon smolts. It is the opinion of this author that combined transportation of live fish and trimmings should be prohibited.

One of the most significant victories for the salmon-farming industry was a reduction in the use of antibiotics realized through good husbandry, preventive hygienic measures, and the development of more efficacious oil-based adjuvant vaccines against certain fish pathogens. Between 1987 and 1999, the salmon industry had managed to reduce its use of antibiotics by 99 percent, and during the same period total salmon production increased eightfold.

Besides supporting a slow-release system for the antigens, oil-based adjuvant vaccines also create a certain nonspecific immunity. This immunity prevails for ISA as well, and several in vivo challenge experiments have documented this protection (Jones

et al. 1999; Anne Ramstad and Odd Magne Rødseth, personal communication). Veso Vikan Akvavet performed experiments in 1997 with an oil-based vaccine that compared results against nonvaccinated controls, which produced a 35-percent relative percent survival against ISA. Later, Intervet Norbio tested their prototype combination ISA vaccine against nonvaccinated fish and found that all of the oil-based vaccines provided relative percent survivals that ranged from 31 to 35 percent (Odd Magne Rødseth, personal communication).

During the first half of the 1990s, vaccine companies competed to improve the efficacy of products, but efforts were specifically directed against furunculosis. This effort resulted in very effective vaccines indeed but also increased the incidence of adverse side effects like abdominal adhesions and melanization (Midtlyng and Lillehaug 1998, Poppe and Breck 1997). Consequently, vaccine companies restructured their strategies and attempted to balance between satisfactory efficacy and acceptable levels of side effects. Some vaccines went too far in the attempt to lower side effects at the clear expense of efficacy. In 1998 and 1999, the salmon industry actually experienced breakdowns in protection against furunculosis for some vaccines. No study was conducted to investigate whether a link existed between the use of specific vaccines and the upcoming ISA situation that occurred from 2000 until 2002. Fish that were harvested in 2000 were evidently vaccinated from August 1998 to April 1999. Statistical information about vaccine sale per product is available from distributors in Norway. Collectively, 13.9 million salmon (Alistair Brown, personal communication) were given doses originating from vaccines linked to lowered field protection against furunculosis.

Although documented by Jones et al. (1999), in this paper it can only be speculated that the nonspecific protection against ISA provided by effective oil-based vaccines reduced the risk of clinical outbreaks and that the use of less-than-efficacious vaccines accordingly increased the risk.

## Conclusion

Recognizing that fish farming is intensive food production on the same scale as that of the poultry and swine industries, we should not be surprised to encounter biological bottlenecks. The emergence of diseases, such as ISA, should be perceived as a biological lighthouse that can be controlled through a combination of management and pharmaceutical and immunological techniques. The disease, therefore, forces legislative and industry personnel to reevaluate current practices and develop standards that will lead to development of biologically balanced and sustainable production in aquaculture.

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# The Eradication of an Outbreak of Clinical Infectious Salmon Anemia From Scotland

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**Abstract:** In 1998, an epidemic of infectious salmon anemia (ISA) occurred in marine salmon farms in Scotland. Eleven farms were confirmed to have infected fish, and a further 25 farms were suspected of containing infected fish. The last suspected case was in November 1999, and the epidemic was officially over on January 16, 2002, when the last farm that shared coastal waters with a suspect case had completed the requisite fallow period. In the European Union, ISA is a List I disease, and a strong legislative framework had already been established in 1993 to bring about its eradication. This framework made the disease notifiable and provided measures to contain and limit

spread and to eliminate the source of infection. On suspect farms and farms in the same coastal waters, the legislation provided for restrictions on the movements of live fish and fish to harvest as well as mandatory biosecurity provisions under the supervision of the Official Service.<sup>2</sup> On farms where the disease had been confirmed, immediate depopulation followed by fallowing and disinfection was required. The average time for withdrawal of all fish in the Scottish epidemic was 21 days and varied in accordance with local logistics and the size of the farm. This, together with the established legal framework to limit spread, was thought to have facilitated successful eradication.

## Introduction

Infectious salmon anemia (ISA) is a contagious and significant viral disease of farmed Atlantic salmon (*Salmo salar*) that first emerged in Norway in 1984 (Thorud and Djupvik 1988). Subsequently, ISA was reported in Canada (initially as hemorrhagic kidney syndrome (HKS) in 1996 (Bouchard et al. 2001, Mullins et al. 1998, Lovely et al. 1999). The disease was then reported in Scotland in 1998 (Rodger et al. 1998, Stagg et al. 2001), the Faroe Islands in May 2000 (Commission of the European Communities 2002), and in the United States [in Maine] in 2001 (Commission of the European Communities 2002). The outbreak in Maine was most likely a consequence of spread from adjacent Canadian waters.

The Scottish outbreak represented the first report of ISA within the European Community. Previously, ISA had not been recorded during official surveillance and testing of farmed or wild fish in the United Kingdom even though the disease has been notifiable since 1990 (Hill 1996). This paper describes the Scottish outbreak and measures taken to eradicate the disease. It also provides commentary on the origins of the infection and the management measures needed for adequate control. Eradication of clinical ISA from Scottish salmon farms was successful, although new legislation was introduced to allow flexibility for rates of depopulation of farms and opportunities for vaccination should

these be needed in the future. If an eradication strategy is pursued, affected farms should be depopulated at the earliest opportunity. Furthermore, given that there is putative evidence for the sporadic occurrence of the virus that causes ISA (ISAv) in farmed and wild fish, preventive measures to thwart emergence of clinical disease are essential.

## Characteristics of ISA

The infectious nature of ISA was first demonstrated in 1987 (Thorud and Djupvik 1988). In early work on the disease, the ability of liver homogenate and infected plasma to transmit the infection was reduced after treatment with diethyl ether and chloroform, suggesting the presence of an enveloped virus (Christie et al. 1993, Dannevig et al. 1995). Knowledge about the etiology of ISA progressed slowly until the virus was isolated in salmon head kidney cells (Dannevig et al. 1995). Electron microscopy and biochemical studies on a Norwegian isolate revealed a single-stranded, enveloped, spherical ribonucleic acid (RNA) virus (Nylund et al. 1995, Dannevig et al. 1995, Falk et al. 1997). Genetic

<sup>1</sup> Dr. Stagg is with the Fisheries Research Services' Marine Laboratory in Aberdeen, UK.

<sup>2</sup> The administration of the fish health legislation in Scotland is a devolved function split between the Scottish Executive Rural Affairs Department (responsible for policy development) and Fisheries Research Services (responsible for administration, enforcement, and surveillance). There is liaison with the U.K. Department of Environment, Food and Rural Affairs, which has responsibility for international relations with the EU and the Office International des Epizooties (OIE).

studies (see the review by Cunningham and Snow in this volume) showed that the ISAv is a multisegmented negative sense RNA orthomyxovirus. Recent classifications showed that there were multiple forms of the virus identified on sequence characteristics of the hemagglutinin (HA) gene and that these probably vary in their pathogenicity (Rimstad and Mjaaland 2002).

Because striking parallels exist with influenza viruses (Webster 1998), particularly important in the management of ISA will be the emergence of new virulent or pathogenic forms of ISAv. Recombination (Devold et al. 2001) and deletion from an ancestral gene (Cunningham and Snow, this volume) have been proposed as mechanisms for the emergence of new strains of ISA in relation to the hypervariable region of the HA gene. The observation of multiple strains, circulating in individual fish (reviewed in Cunningham and Snow's paper) enhances the likelihood that these mechanisms occur. These characteristics also have implications for the relative importance of vaccination and eradication as control measures.

Disease outbreaks appear to be associated with highly infectious forms of the virus transmitted via live fish, urine, feces, skin mucus, and dead fish. The virus adheres to red blood cells through attachment via the HA protein. In virulence experiments (Totlund et al. 1996), blood homogenate is the most infectious inoculum, and passive transmission is assisted by virus-contaminated waste, such as blood in slaughterhouse effluent. This finding emphasizes the need for satisfactory disinfection practice in relation to fish farms and slaughterhouse waste and effluent to reduce the risk of ISA transmission. Sea lice have been suggested to be a vector for transmission (Rolland and Nylund 1998) because they feed on the blood and tissue of their host. The disease appears to be restricted to marine salmon farms. Several factors are important in determining whether or not fish at a seawater salmon site develop clinical disease (Jarp and Karlsen 1997):

- Proximity to another ISA-affected farm,
- Proximity to untreated processing effluent,
- Sites that exchange fish between processing and growout areas,
- Multigeneration sites,
- Poor farm hygiene (especially bleeding at sea), and
- Sites with multiple sources of smolts.

## **Management of the Epidemic**

ISA is a List I disease in the European Union and is managed under the auspices of two European Commission (EC) directives governing trade in aquaculture products (Commission of the European Communities 1991) and control of fish diseases (Commission of the European Communities 1993). These require that the management of ISA in Scotland be based on three elements:

- Passive surveillance to identify the first occurrence of the disease at the earliest opportunity,
- Application of biosecurity measures to prevent spread, and
- Elimination of the source of the infection.

Within the legislation, action is required if ISA is suspected to be present among fish on a farm and if investigation of suspicion results in confirmation of the presence of the disease. In Scotland, farms were confirmed if fish in the farm showed clinical disease characteristic of ISA (i.e., clinical signs, histopathological lesion(s) pathognomonic of ISA, anemia) and there was evidence of infection with ISAv by culture, reverse-transcriptase-polymerase chain reaction (RT-PCR) assay, or the indirect fluorescence antibody test (IFAT). Suspect farms had fish with evidence of infection, but tests to prove the presence of clinical disease were inconclusive. Subsequent changes to the legislation now require that a site be confirmed if ISAv is isolated in two samples from one or more fish at the farm tested on separate occasions.

## Notification and Surveillance

Since 1991, ISA has been notifiable to the Official Service in the United Kingdom. This measure is supported by official inspection and sampling (once a year for marine sites) to guarantee continued freedom from viral hemorrhagic septicemia (VHS) and infectious hematopoietic necrosis (IHN). Experience shows that, although surveillance confirms the absence of an infectious agent, it has not been particularly useful at detecting new outbreaks. For example, initial outbreaks of VHS (Munro 1996), ISA (Rodger et al. 1998), and sleeping disease (Branson 2002) were first identified through notification of the disease or investigations of abnormal mortality rather than detection by official surveillance. It follows from this that an important aspect of risk reduction is that notification be mandatory and that there be good relations between the Official Service and industry to promote reporting. Following the initial outbreak, the Official Service then carried out an enhanced surveillance program in the first instance over a wide area (40 km from the affected sites) and subsequently in focused surveillance zones around suspect and confirmed sites. This surveillance, aided by contact tracing, did detect new secondary outbreaks and was an important aspect of bringing the disease under control.

## Measures To Prevent Spread

U.K. authorities already had strong enabling legislation to implement the EC directive for the control of fish diseases (European Commission 1993). This situation allowed a range of measures to be put in place immediately once the disease was reported to the Official Service. From the outset, the objective of the control regime was to contain and eradicate the disease. On suspect farms and farms sharing coastal waters with confirmed and suspect farms, these measures included

- Preventing the movements of live fish other than in exceptional circumstances when the risk of allowing a movement was less than the risk of fish staying in situ;
- Placing conditions on the movements of dead fish, people, and materials liable to transmit the disease to minimize the risk of transmission; and
- Fallowing and disinfecting the farm to break the cycle of infection.

In addition, complete and immediate depopulation of all fish from confirmed farms was required. Such fish could be harvested for sale. If the fish proved unsuitable for harvest or showed clinical signs of disease, they were destroyed.

Despite this legislative framework, further development of the decisionmaking process was required to manage the outbreak. A rational basis for defining the area of the coastal zone affected by the disease was required as well as a regime to determine the fallowing period necessary within this coastal zone.

## Determination of the Area of the Coastal Zone Affected

Farms near confirmed or suspect farms were at risk of contagion through local spread (Murray, this volume), but the zone concept as it related to coastal marine waters was poorly developed when the ISA epidemic occurred. There was little information about the mechanisms of ISA transport by passive dispersion. Hydrographic data on individual sites were not easily available, nor was there sufficient time to survey sites or model dispersal patterns around individual farms. It was rapidly realized that these constraints and a complex coastline meant that a simple and robust method would be needed to identify adjacent farms at risk using existing site locations and available generic hydrographic information.

During the epidemic, some basic information was available on the mechanism underlying horizontal transmission of ISA. Some researchers

had drawn the conclusion (Nylund et al. 1994) that passive transmission through seawater would be negligible because of the potential dilution. This argument was not supported by information on shedding rates, dilution, and the viral dose required to infect susceptible fish. It is now understood that the infectious dose for ISA is low (Raynard et al. 2001b) and that local diffusive processes explain the spread within some sea lochs in Scotland (Murray, this volume).

To establish control and surveillance zones, it was necessary to determine the distance over which infection from a suspect or confirmed farm posed a risk to adjacent farms (Turrell et al. 1998). The hydrographic information used for such purposes was the maximum spring tide current obtained from a tidal atlas (Lee 1981). This information was translated into tidal excursions ( $Xt$  in meters) around each fish farm from the formula  $UT/\pi$ , where  $U$  = tidal current amplitude ( $\text{ms}^{-1}$ ),  $T$  = tidal period (12.42 h or 44,712 sec), and  $\pi$  is a constant (3.1416). Most salmon farms are located in relatively sheltered locations, and therefore the maximum tidal amplitudes were restricted to  $0.51 \text{ ms}^{-1}$  (1 knot) in mainland Scotland and  $0.255 \text{ ms}^{-1}$  (0.5 knot) in Shetland, giving tidal excursions of 7.3 and 3.6 km, respectively.

The tidal excursion defines the region around a farm that is potentially infected on the basis of tidal transport alone. When tidal excursions from adjacent farms overlap, there is a risk that infection may be transmitted from one farm to another with contiguous tidal excursions. Transmission from one farm to another under this model will be broken only when there is a gap large enough to ensure that adjacent tidal excursions do not overlap (i.e., when farms are separated by a distance greater than 2 tidal excursions). The area enclosed by contiguous tidal excursions formed the surveillance zone around farms. Extra surveillance, by the Official Service, and fallowing requirements were necessary in this area because of the perceived risk of infection becoming established. All farms bounded by the tidal excursion of a confirmed or suspect farm were also required to coordinate their fallowing to minimize the likelihood of

recurrence. An example of the tidal excursion model applied to define zones around suspect farms is given for a typical Scottish sea loch in figure 1.

The tidal excursion model accounts for waterborne tidal infection only and does not consider residual flows. Waterborne infection includes viral particles in the water column or bound to particulate material (including blood) or sea lice but cannot explain transmission by free-swimming fish. The virus has been detected in wild sea trout (*Salmo trutta*) (Raynard et al. 2001a), saithe (*Pollachius virens*) from infected farms (Snow et al. 2002), and escaped Atlantic salmon (Raynard et al. 2001a). Although some of these species migrate over large distances, transmission by wild or escaped fish

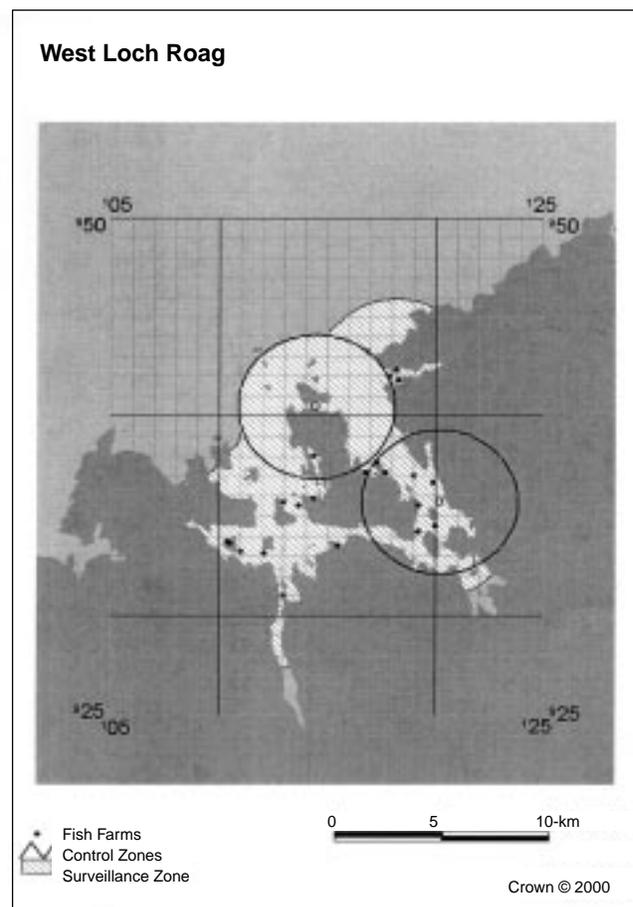


Figure 1—An example of the type of surveillance and control zones established during the course of the Scottish ISA epidemic.

vectors was not considered to contribute significantly compared with anthropogenic spread in the Scottish epidemic (Murray, this volume). The output of the tidal excursion model also compares favorably with the results of a Norwegian case control study (Jarp and Karlsen 1997). This study established an inflection in regressions of probability of infection against distance at 5 km from an infected farm. Analysis of all RT-PCR data reporting ISA infection in salmon farms following the Scottish outbreak (Stagg et al. 2001) also showed that the majority of RT-PCR signals came from within the surveillance zones of the tidal excursion model established to contain the disease. It is possible that the tidal excursion model may not apply to other coastal areas. If so, individual assessment of its usefulness within different tidal regimes would be required. The model as applied to managing the outbreak of ISA in Scotland, however, contributed greatly to the successful control of the epidemic. It also formed a central part of the risk management to reduce the likelihood of recurrence of ISA in Scotland by providing a rational basis for the subdivision of the coastal zone in which area management agreements and codes of practice (Joint Government/Industry Working Group on ISA 2000a) can be implemented.

## **Rationale and Risk Assessment for Restocking Following the Outbreak**

Key decisions in the management of an infectious disease in a commercial animal-rearing system are the measures taken to prevent residual infection that could trigger a further epidemic following restocking. ISA affects salmon in the marine phase or in hatcheries using seawater. There is some evidence that ISA occurs in freshwater, but it is sporadic and at a low level (Raynard et al. 2001a, Stagg et al. 2001). Thus, salmon smolts introduced to a new sea-cage site will be susceptible, and any residual infection with ISA may trigger a new outbreak.

Management of residual infection in sea cages requires the cleaning and disinfection of all structures that may have been in contact with fish or contaminated equipment in addition to fallowing the site. Cleaning and disinfection requires dismantling the cages and transporting them to shore for cleaning and disinfection. Nets usually need to be transported to a net-washing station in a skip or other container to prevent leakage.

Following the Scottish epidemic, concrete barges and other large structures were cleaned to the water line, disinfected, and fallowed onsite. Farmers were required to clean and disinfect by (1) scraping and brushing to remove all gross fouling and organic matter; (2) cleaning and hot washing to remove remaining particulate matter, fats, and oils that were likely to bind the virus; and (3) a final wash with a proprietary disinfectant. Major considerations in the selection of disinfectants were efficacy and impact on the environment.

Fallowing of a site minimizes the recurrence of disease from environmental reservoirs (sediment, water, fish, and shellfish). Specific benefits of fallowing have been shown for control of sea lice infestations (Bron et al. 1993, Grant and Treasurer 1993), but results were somewhat equivocal for microparasites (Jarp et al. 1995, Wheatley et al. 1995). Nevertheless, there is fair agreement among industry and regulatory experts on the benefits of fallowing and year-class separation (Stewart 1998). The practice of fallowing marine cage sites was a major factor in reducing the incidence of ISA in Norway from its peak of 101 cases in 1990 to somewhere between 2 and 15 cases per year between 1994 and 1998 (Håstein 1997, Håstein et al. 1999). The Norwegian experience showed that a 6-month fallow period was sufficient to prevent recurrence of ISA following an outbreak. In the Scottish epidemic, however, fallowing strategies also needed to be developed for farms containing fish suspected of being infected and farms within the control and surveillance zones. A precautionary approach was initially adopted based on the

Norwegian experience (table 1). Some suspect farms proceeded to harvest with little additional evidence of infection despite regular monthly surveillance by the Official Service. Fish on farms where this occurred typically gave positive results for IFAT and RT-PCR assays but did not show clinical signs of ISA. A risk-based approach to fallowing suspect sites was therefore developed based upon two assessments: the risk of a farm's carrying infection at the time of harvest and the risk of transmission to an adjacent farm.

The infection risk was assessed by a review of the surveillance results for a particular farm in the 4 months immediately before harvest. Those farms with high risk were those that, in the last 4 months, either had evidence of infection sufficient to designate the farm suspect or were within the tidal excursion of a farm that had been declared to be suspect. Farms containing fish with a lower threshold of infection with ISA were categorized as being at medium risk, and those without any subsequent evidence of infection were designated low risk. A monthly surveillance program that tested moribund fish was used to assess the infection status and was considered more reliable than a single random sampling at harvest.

The transmission risk was assessed as low, medium, or high according to the likelihood of

transmission from the suspect farm to others in the vicinity. The level of risk was determined by consideration of

- Physical factors, such as the local hydrography and topography influencing the separation of sites;
- Biological factors, such as evidence of the transmission of infection to wild fish; and
- Industry factors, such as the density of farms, the use of common facilities such as shore bases, and other evidence of site-to-site interaction.

Infection and transmission factors were combined to provide a fallowing period that varied on a sliding scale from 3 to 6 months (fig. 2).

## Removal of the Infection Source

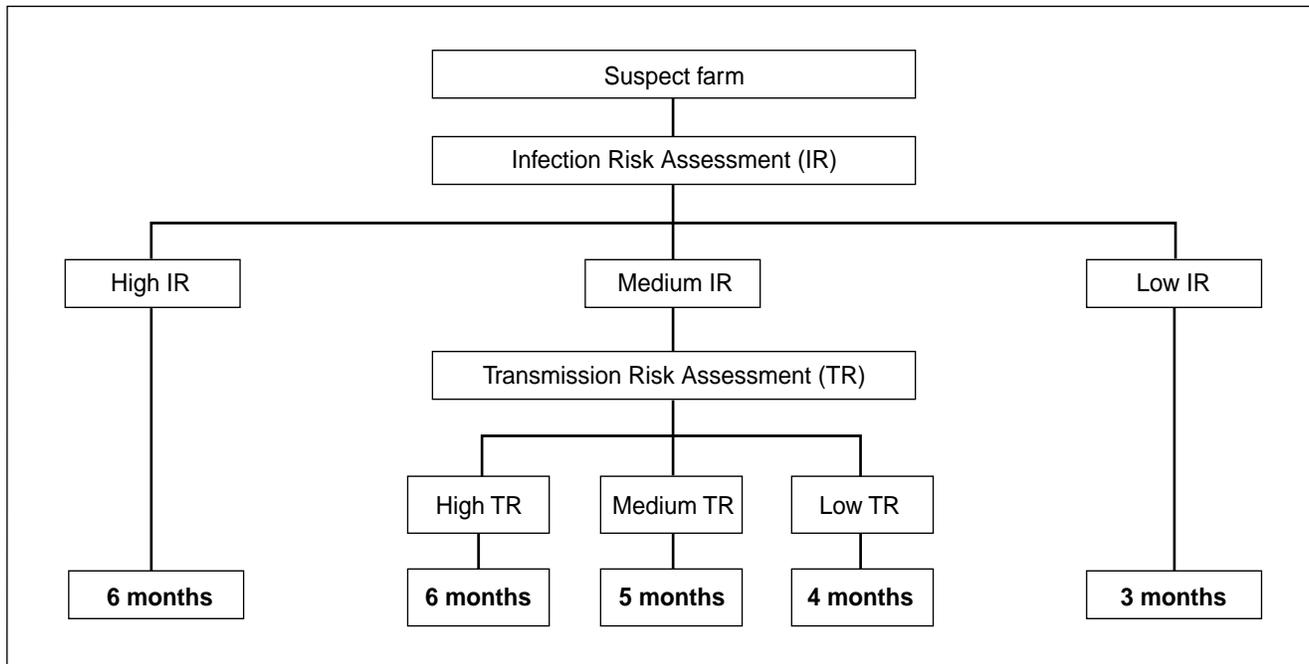
In Scotland, legislation required immediate depopulation of fish from farms in which the disease had been confirmed. In reality, this was accomplished within the shortest practical time after consideration of the logistics required and the tonnage of fish on a site. Farmers were allowed to harvest fish from sites if they did not show clinical signs of disease. Fish that were either unsuitable for harvesting or had clinical signs of ISA were culled, and the virus was inactivated by ensiling in formic acid to a pH less than 4.0.

During the epidemic, flexible schemes were discussed based on the Norwegian experience of depopulating only individual cages on infected farms if the mortality, attributed to ISA, approached 0.05 percent per day (Hastein 1997). This approach had been successful in reducing the prevalence of ISA in Norway but failed to eliminate new outbreaks. The Control Directive (European Commission 1993) was therefore modified (European Commission 2000) to allow phased depopulation and the use of vaccination programs to help eradicate the disease.

The average time for depopulation of infected farms in the Scottish epidemic was 21 days. This rapid rate of depopulation is likely to have been an

**Table 1—Fallowing regimes used to prevent recurrence of infectious salmon anemia following the 1998 epidemic in Scotland**

Category of farm	Initial fallow period: May 1998–December 1999	Fallow period after risk assessment: after December 1999
Confirmed Suspect	6 months 6 months	6 months 3–6 months based on risk assessment
Uninfected farm in control zone	3 months synchronously with confirmed or suspect farms	6 weeks synchronously with confirmed or suspect farms
Uninfected farm in surveillance zone	6 weeks	6 weeks



**Figure 2**—Decision tree for following a salmon farm containing fish suspected to be infected with ISA.

important factor in the successful eradication of ISA from Scottish farms compared with other countries where cages not suffering mortality or clinical disease have remained until harvest and epidemics have persisted.

## The Scottish Epidemic

In Scotland, the first case of ISA was reported in Loch Nevis in May 1998 (Rodger et al. 1998), and another 10 cases were subsequently confirmed (Stagg et al. 2001) extending over the whole of Scotland, including the Shetland Isles (fig. 3). Twenty-five farms contained fish suspected of being infected, but the disease did not progress sufficiently for it to be confirmed. The incidence of new cases (farms) peaked in April 1998 (fig. 4), and the last case was confirmed in May 1999, 1 year after the initial outbreak. Suspect cases were investigated until November 1999, and scientific experts have independently drawn the conclusion that clinical

cases of ISA have been eradicated from Scottish salmon farms (Royal Society of Edinburgh 2002).

Investigations revealed that the epidemic arose from a single primary site located in Loch Nevis on the Scottish west coast (Stagg et al. 2001). This was based on several lines of evidence:

- The pattern of spread of the disease was discontinuous, and intervening unaffected areas subsequently became infected (Murray, this volume).
- Virus isolates obtained from farms with clinical diseases (5 out of 11 confirmed farms in the epidemic) were identical in terms of the observed sequence for RNA segments 2, 8, and 6 (Cunningham and Snow, this volume).
- All confirmed sites and most of the suspect sites had significant epidemiologic connections to the primary site at Loch Nevis (Stagg et al. 2001). Two of the subsequently affected areas (Loch Creran and Loch Snizort on Skye) had received live fish from the primary site. Another affected site in

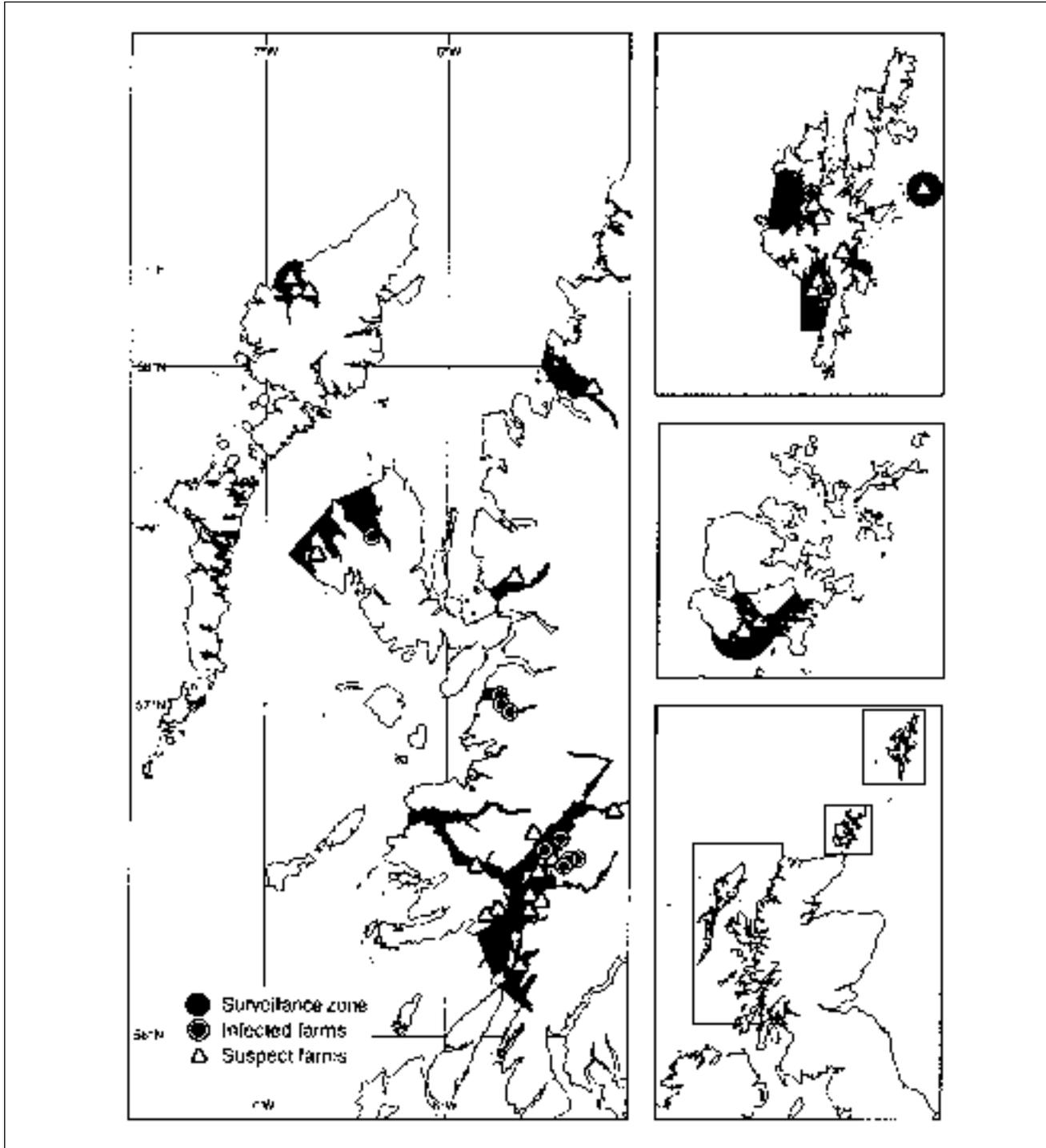
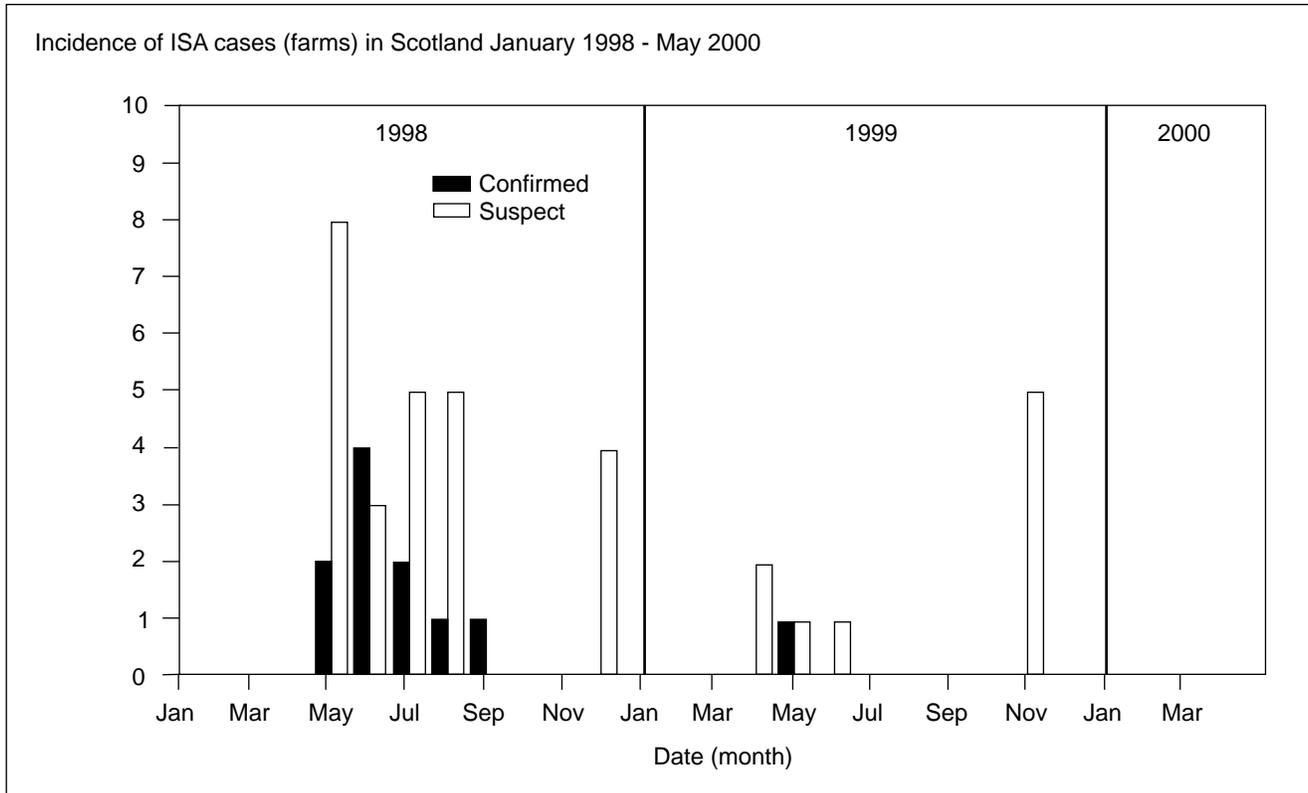


Figure 3—Location of ISA suspect and confirmed farms in Scotland, 1998–99.



**Figure 4**—Incidence of ISA cases among salmon farms in Scotland from January 1998 until March 2000.

Shetland had been stocked with smolts from a remote site on the Western Isles, but these smolts had been delivered by transit through the site in Loch Snizort.

(d) Site-to-site movements of wellboats played a significant role in the spread of ISA that was mainly associated with trips to harvest (Murray et al. 2002). The spread from the primary site was also associated with equipment transfer for grading or harvest of fish, sharing divers and their equipment, mixing clean with dirty nets at net-washing stations, and transport of dead fish (mortalities) for disposal (Stagg et al. 2001). A most significant epidemiologic factor associated with the broadcast of ISA away from the primary site was the linkage to harvesting via wellboats through a harvest station. Harvest stations are used for the temporary holding of fish before slaughter. These stations are sea

pens anchored next to a slaughterhouse and processing plant. The creation of such harvesting stations in combination with the trafficking of wellboats between rearing sites and the harvest station itself were identified as the highest risk factors associated with the transmission of ISA over distant geographic regions (Munro et al. 2003).

(e) Other marine sites that had been stocked with fish from the same freshwater populations used to stock the Loch Nevis sites did not simultaneously have outbreaks of disease. Although some (not all) did subsequently show evidence of the disease, the most likely cause was established contact with the primary occurrence in Loch Nevis. In addition, siblings and contacts still in freshwater that were tested did not show any evidence of infection.

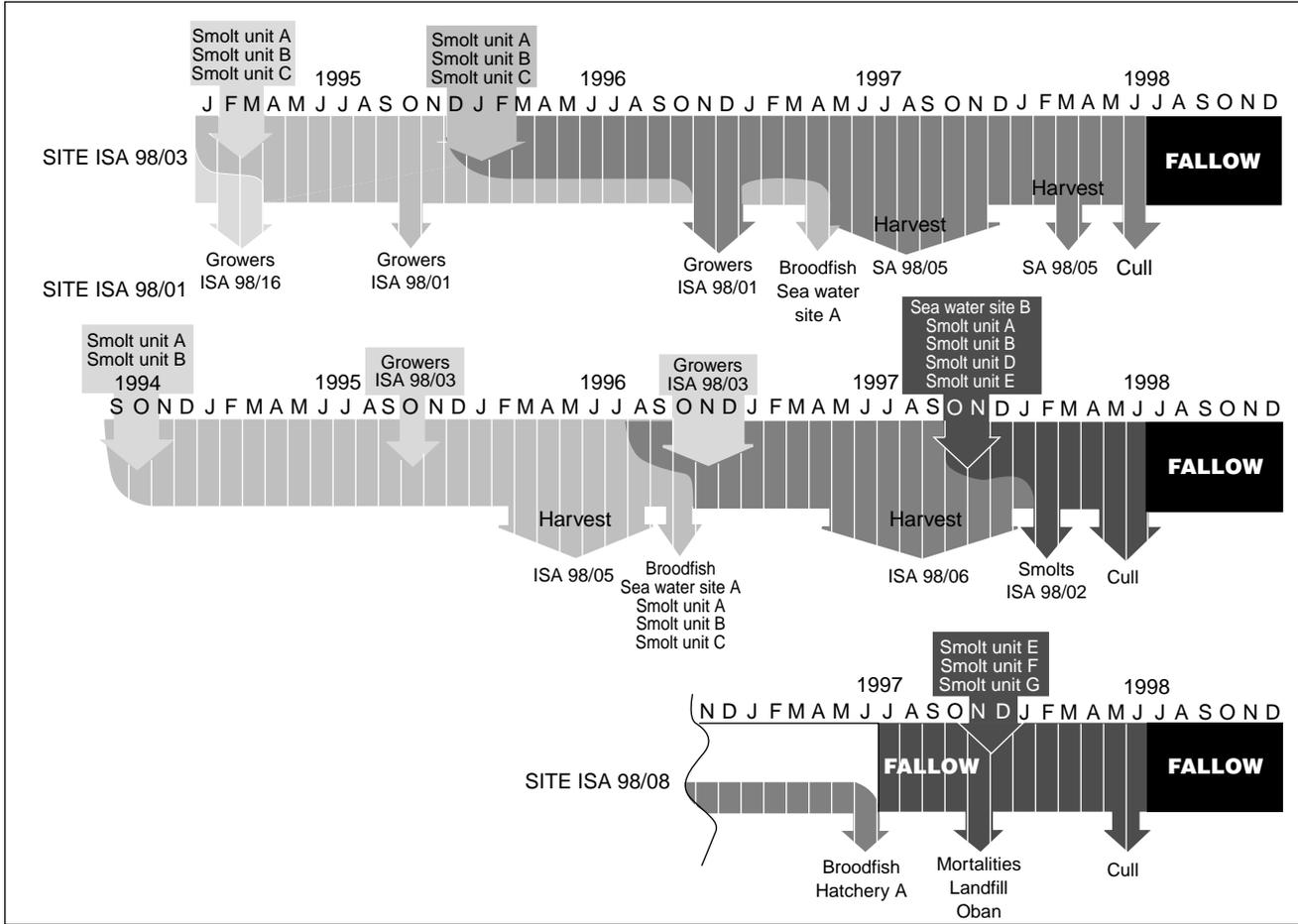
These findings suggest that the epidemic arose at the primary site and was disseminated by the

actions of fish farmers. However, it was also evident that an epidemiologic link to a confirmed farm was no guarantee that the fish in a recipient farm would develop clinical ISA. When this occurred, the reproductive ratio ( $R_0$ ) must have been greater than unity (Anderson and May 1991). The size of  $R_0$  will be dependent on the dose of ISAv required, whether conditions on a farm promote transmission of the disease, and the numbers of infected and susceptible fish on the farm. The incubation of the disease in relation to the time from the introduction of infection to the time to harvest was also critical in whether disease manifested. On two farms where the introduction of infection was actually determined, the incubation time was about 3 months (Stagg et al. 2001). This figure compares with an incubation time of around 21 days when Atlantic salmon were experimentally challenged with ISAv (Raynard et al. 2001). In the Scottish epidemic, 19 of 25 suspect cases had clear evidence of infection and significant epidemiologic connections to the primary site (Stagg et al. 2001), but their fish did not develop clinical disease before harvest. Fish from a farm in Shetland did not develop clinical disease despite isolation of the virus that was genotypically identical (segments 2, 6, and 8) to that isolated at the primary site.

An alternative explanation for the pattern of ISA outbreaks in Scotland is that the disease arose sporadically from a background wild source. Surveys carried out in Scotland showed that ISAv can be detected sporadically in wild fish by RT-PCR (Raynard et al. 2001a)—often in areas remote from the infected farms. The virus was also detected by RT-PCR in fish from some farms outside the surveillance zones established to combat the disease (Stagg et al. 2001), and five of the suspect farms did not have any evident connection to the primary outbreak. However, it is clear that these observations are insufficient to explain the strong linkage established between infected sites adequately.

## **Origins of the ISA Epidemic**

It has not been possible to identify the source of ISAv that triggered the epidemic in Scotland definitively. Assessment of the current evidence indicates that the virus was either imported from infected areas in Norway by traffic of contaminated wellboats or emerged from a benign infection among wild fish (Stagg et al. 2001). Since 1999, ISAv has been detected by RT-PCR in both farmed and wild fish. In farms, the monthly mean prevalence of infection has declined dramatically over the course of the epidemic. Although ISAv should have disappeared according to the trend in these data, it was still found on isolated occasions (Stagg et al. 2001). ISAv was also occasionally detected among fishes by RT-PCR beyond ISA control zones during the epidemic and among wild salmonids throughout Scotland (Raynard et al. 2001a). In none of these cases was ISAv associated with evidence of disease. This fact suggests that ISAv might exist within a widely distributed wild host having the potential to transmit the virus to more susceptible hosts if conditions are suitable. This supposition implies a continual, albeit rare, risk of reemergence. The primary site of the first occurrence of ISA in Scotland was characterized by high stocking densities, multiple seawater-to-seawater movements of live fish, continuous stocking, and multiple generations (fig. 5). These conditions give maximum opportunity for the emergence of a virulent pathogen.



**Figure 5**—Stocking regime at farms in Loch Nevis before the outbreak of ISA. Site 98/01 was the primary source of the outbreak.

## Implications for Future Management

The experience of the ISA epidemic in Scotland had some unforeseen consequences. Industry and government established a working group to address the risks posed by ISA (Joint Government/Industry Working Group 2000b). The practical consequence of this work was the development of an industry code of practice (Joint Government/Industry Working Group 2000a) to prevent emergence of new outbreaks and their spread to other farms. The code of practice covers fish and equipment movements,

area management by the tidal excursion model, harvest and processing practices, and the disposal of carcasses and waste. Although principally designed to prevent the spread of disease, the code of practice provides guidance on the adoption of single-year-class strategies and fallowing. Applied to management zones, these measures also preclude emergence of ISA.

The development of ISAv vaccines (Brown et al. 2001) may provide another method for the control and prevention of ISA. In many ways, vaccination is preferred to the current practice of depopulation of farms because of the evident economic benefits.

However, a vaccination strategy should be pursued cautiously. Killed-cell vaccines may interfere with current nonculture-based diagnostic methods and complicate differentiation between vaccinated and infected populations. Vaccine research needs to establish that vaccines prevent carrier status as well as clinical disease and can confer protection against the wide range of strains of ISA identified thus far (Cunningham and Snow, this volume). Regulators and farmers need to be confident that the development of vaccines against one strain of ISA will not merely increase the evolutionary pressure for the emergence of new strains resistant to the vaccine.

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# Practical Grower Experience in the Proactive Prevention and Control of Infectious Salmon Anemia

Sebastian M. Belle<sup>1</sup>

**Abstract:** Clinical infectious salmon anemia (ISA) was first reported in Maine waters in 2001. Maine salmon growers have developed and implemented both proactive and reactive responses to it. Annual third-party biosecurity audits were initiated in 1997. These audits proved to be a valuable learning tool for farm managers and greatly assisted in the identification of higher risk farm practices. In 1998, an ISA action plan was developed and implemented by the growers with the help of a number of fish health professionals. This plan was revised annually based on the evolving state of knowledge about ISA epidemiology, improving ISAv detection methodologies, and experience in ISA management from other salmon-producing areas facing ISA outbreaks. In 2001, the Maine Aquaculture Association (MAA) developed and implemented a cooperative bay management program designed to increase grower coordination and reduce risks associated with fish health

and environmental impact. The MAA Bay Management Agreement was based on the most recent MAA ISA Action Plan and systematic review of cooperative bay management plans and biosecurity protocols from around the world. The Agreement establishes minimum standards, procedures, and protocols designed to minimize disease risk and improve grower communications and cooperation. MAA has worked closely with State and Federal authorities to ensure that grower practices and responses are consistent with the best available scientific guidance in aquatic disease prevention and response. The U.S. Department of Agriculture's (USDA) ISA Program has been critical to the rapid and aggressive response to the emergence of clinical ISA in U.S. waters. Continued close cooperation between Federal and State authorities and the growers will significantly increase the effectiveness of any ISAv control and eradication efforts.

## Introduction

Infectious salmon anemia (ISA) was first detected in Norway in 1984 (Thorud and Djubvik 1988, Hovland et al. 1994, Hastein 1997). Subsequently, the disease has been detected in the Canadian provinces of New Brunswick (1986) and Nova Scotia (1998) (Bouchard et al. 1999, Lovely et al. 1999), Scotland (Rodger et al. 1998, Rowley et al. 1999), Chile (Kibenge et al. 2001), and the Faroe Islands in 1999 (Anonymous 2000). Some evidence suggests that ISA virus (ISAv) has been present in North America for some time, predating actual ISA outbreaks (Krossøy et al. 2001).

Clinical ISA was first reported in Maine in March 2001. The Maine Aquaculture Association (MAA) and its member growers have worked closely with State and Federal authorities and fish health professionals to ensure that grower practices and responses are consistent with the best available scientific guidance and in compliance with State and Federal environmental and fish health regulations. Beginning in 1997, Maine salmon growers developed and implemented a series of proactive and reactive responses to the threat of ISA. These actions were based on a philosophy of avoidance of disease

through sound fish health and husbandry practices and on the guiding principles for management of infectious diseases—risk reduction, detection, containment, and control through selective eradication.

Grower initiatives have taken a number of forms: (1) systematic reviews and revisions of husbandry practices and farm operations to reduce fish stress, (2) increased fish health surveillance and testing, (3) ISA vaccine trials, (4) third-party biosecurity audits, (5) fish health action plans, (6) cooperative bay management agreements, and (7) assistance in the development of the ISA program and program standards by the U.S. Department of Agriculture (USDA). This paper will discuss only initiatives 4–7.

## Biosecurity Audits

An industrywide program of annual, third-party biosecurity audits was initiated in 1997. Biosecurity audits are proactive tools that use disease risk-analysis techniques to systematically identify, characterize, and measure sources and levels of risk. The audit characterizes by qualitative and quantitative examinations the reality of how a company and its workers implement biosecurity protocols, not theoretical plans or company policies. Auditors specifically attempt to identify weaknesses

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in site-specific biosecurity practices. The audit format and methods have undergone a number of revisions since their inception. Audits are conducted by licensed and accredited veterinarians who are independent subcontractors working for the Maine Department of Marine Resources or USDA. Auditors conduct unannounced visits to marine farms, hatcheries, processing plants, and transportation boats. Auditors are given uninhibited access to make direct observations and interview employees to determine the level of relative risk associated with operational practices, facility design, and layout. Each specific area examined (up to 121 different areas depending on the type of facility or vessel) is classified as no/low, low, moderate, or high risk. Audit areas that are seen as particularly affecting disease risk (35–45 factors) are given additional weight during the audit analysis. Facility-specific audit reports are sent to the individual farmer, and an annual, comprehensive report, which characterizes industrywide risks and allows comparisons between farms, is sent to both the Department of Marine Resources and the growers' association.

Biosecurity audits have proved to be a valuable learning tool because they directly identify specific farm practices that increase disease risk and include recommendations on how to improve operations and reduce risks. The power of the biosecurity audit as a disease management tool stems from its systematic approach, utilization of external auditors, repeatability, and direct feedback to farm managers. Subsequent audits allow both regulators and growers to determine if operational biosecurity is improving, staying the same, or decreasing. In general, variances in performance between farms have decreased over time, and audit results have improved significantly since the program's inception (Allen and Opitz 1999, Merrill 2000 and 2001). While biosecurity protocols and audits can be very effective preventative tools, management intensity and focus are critical to creating and maintaining a climate of constant vigilance and prevention. Effective risk control mandates that biosecurity becomes an ingrained set of operational habits that are constantly reassessed and improved.

## **Action Plans**

In 1998, with the help of a number of fish health professionals, an ISA action plan (ISAAP) was developed and implemented by the growers (Maine Aquaculture Association 1998). The ISAAP is a dynamic document designed to be periodically revised to incorporate the evolving knowledge about ISA epidemiology, improved ISA detection methodologies, and increased level of experience in ISA management. Formulation of the ISAAP served as a catalyst for increasing grower cooperation and generating support for USDA involvement in aquatic animal health management. In order to come to critical consensus, growers learned to think cooperatively and develop mechanisms for acting cooperatively as they were forced to review their own and each other's operational practices and examine how they affected disease risks. This new cooperative approach was instrumental in the development of grower initiatives for comprehensive bay management planning. Thus the ISAAP formed much of the basis for the MAA Finfish Bay Management Agreement (FBMA) that was signed in January 2002 (Maine Aquaculture Association 2002).

The ISAAP outlined a series of strategic recommendations from the Maine salmon farming industry for dealing with containment and control of ISA and ISA and documented the industry's approach to rational management of ISA through husbandry and biosecurity practices. Some of the specific biosecurity protocols and operational standards recommended in the ISAAP included

- Developing veterinary–client relationships for all farms and facilities to achieve regular onsite fish health evaluations whose frequency would increase should unusual or sustained mortality occur;
- Initiating specific sampling protocols with increased sampling in an “ISA Increased Risk Zone”;
- Initiating standardized third-party biosecurity audits at all farms and transport and processing facilities;
- Imposing severe restrictions on fish movement from sites with confirmed ISA-positive status;

- Developing single year-class stocking and production strategies;
- Initiating sea-lice treatment programs in accord with existing State of Maine programs;
- Implementing guidelines on the containment, disinfection, and disposal of all forms of waste associated with farm mortality and harvesting and processing operations;
- Implementing strict site, equipment, and personnel disinfection guidelines and specific recommendations on intersite boat, equipment, and personnel traffic; and
- Promoting specific recommendations for priority research to answer critical questions about ISA management.

Although the ISAAP contained a number of specific recommendations for operational standards and biosecurity measures, it was also a policy document. In particular, the ISAAP articulated the industry position on what constituted a positive test result and how those results should be used in the classification of the disease status of an individual fish, cage/tank, and/or farm site. The plan further clarified the growers' perspective on these definitions and how they related to reporting requirements. The plan highlighted the importance of indemnification and its linkage to effective disease management. Finally, the ISAAP proved to be a very effective tool in lobbying for USDA involvement in addressing the ISA issue.

## Cooperative Bay Management Agreement

To date, ISA has not been eliminated in any of the countries that have experienced clinical outbreaks. Given the many factors involved in disease transmission, elimination of ISA may not be an attainable goal. Instead, loss reduction and risk minimization through effective management techniques such as stringent surveillance and aggressive biosecurity protocols may be more realistic.

In 2001, the MAA developed a Cooperative Bay Management Program designed to increase grower coordination and reduce risks to fish health and the environment associated with intensive fish production. The MAA Finfish Bay Management Agreement was based on the most recent MAA ISAAP and a systematic review of cooperative bay management plans and biosecurity protocols from around the world. Implementation of the agreement began in January 2002.

The agreement is an overarching, *legally binding* document signed by all private salmon farmers in the State of Maine. The agreement establishes minimum standards, procedures, and protocols that are designed to lessen disease risk and improve grower communications and cooperation. Signatories to the agreement are obligated to establish Local Bay Management Groups for all eight local bay management areas in Maine. These groups of farmers are in turn obligated to develop a local bay area management plan, which is based on the minimum standards and guidelines established in the overarching agreement and which is then reviewed and approved by the agreement signatories. Local plans may establish more stringent standards and protocols than those contained in the agreement.

The agreement includes a series of technical appendices and a set of specific definitions for terms to be used in both the agreement and local plans. The agreement also contains a series of legal terms and conditions that refer to meeting frequency, amending procedures, arbitration, governing law, and the relationship between the signatories. The term of the agreement is linked to production cycles in saltwater farms and is automatically renewed. Signatories to the Agreement commit to comply with all relevant State and Federal regulations and to all collective industry standards. The agreement is *not* intended to coordinate production or business strategies for the purpose of price or market influence, and great care was taken to ensure the agreement did not violate antitrust laws.

The Agreement contains a generic bay management template for use when developing local plans. This template establishes the minimum standards and protocols that local plans must include and begins with a common mission statement:

As marine farmers, we have a strong vested interest in healthy marine ecosystems and a clean marine environment. Our mission is to achieve long-term viability and competitiveness in the Maine Salmon Farming Industry with a continued commitment to environmental sustainability and stewardship. We seek to promote responsible development and management of the Maine Salmon Farming Industry in order to assure the production of high quality food while respecting environmental considerations and consumer demands. (Maine Aquaculture Association 2002)

The local plan template goes on to identify the specific geographic coordinates and individual farm occupants of each local bay management area. A long-term stocking and production target for the local area is established, and any constraints inhibiting its attainment are identified. Specific actions required to overcome these constraints are identified, and a timetable for achieving the long-term target is established. Although the template includes 14 focus areas, the 5 principal sections of the local plans address communication, waste management, pest management, fish health management and biosecurity, and disinfection.

## **Communication**

Local bay management groups must develop a Bay Management Area Communication Plan (CP) that clearly outlines individual and collective responsibilities and methods to facilitate rapid and clear communication within and between bay

management groups. The CP must include methods designed to reduce the risk of misinformation being distributed. The CP establishes risk thresholds that trigger communication among producers in each local bay management area. These thresholds must recognize that different risks engender different potential impacts. Lower thresholds are established for risks that have higher potential impacts. The CP establishes the baseline information that must be communicated about disease risks on any individual farm, e.g., history, character, prevalence, and potential for dissemination of the identified risk; specifics of fish species affected; and actions taken to control and contain the risk.

## **Waste Management**

A Bay Management Area Waste Management Plan (WMP) must be developed. This plan must require all farm occupants in a bay management area to develop site-specific WMPs that are reviewed and approved by the Bay Management Group. The WMP must clearly identify all wastes generated and classify them with respect to any risks associated with their collection and appropriate disposal. Whenever possible, the WMP should encourage reduction, reuse, and recycling of waste. WMPs must address, at a minimum, human waste, feed bags, scrap rope and netting, fish mortalities, packaging materials, and any chemical or fuel spills. The WMP is applied in concert with the specifics of the Fish Health and Biosecurity Plan (below).

## **Integrated Pest Management**

All local Bay Management groups must develop a Bay Management Area Integrated Pest Management Plan (IPMP). This plan requires all operations in a local bay management area to coordinate their efforts at pest monitoring and control and to use best management practices to reduce the need for use of chemicals or medications. At a minimum the IPMP includes coordinated monitoring and treatment, single year-class stocking, fallowing between year-classes, pest population thresholds for

treatment decisions, and treatment withdrawal guidelines.

### **Fish Health and Biosecurity**

All local Bay Management groups must develop a Fish Health Management and Biosecurity Plan. This plan provides guidelines and protocols intended to reduce the risk of the introduction and spread of infectious agents (e.g., ISA<sub>v</sub>) due to human activities. The plan requires all farms, transport vessels, and processing facilities in Maine to have regular third-party biosecurity audits. Facilities that are either confirmed positive for fish with ISA, process fish from sites confirmed positive for ISA, or have consistently bad audits shall be subject to biosecurity audits more frequently. Husbandry and handling protocols are also established for all life-history stages. In coordination with the WMP, the Health Management and Biosecurity Plan establishes strict guidelines for the collection, storage, and disposal of farm mortalities and blood water. The plan establishes specific protocols for appropriate use, movement, and disinfection of divers, grading, net changing, and harvesting equipment. Personnel and boat movement protocols and disinfection standards are also included. Vessels are required to declare a “home” bay management area with movements between bay management areas highly restricted and dependent on thorough disinfection. Vessels moving between sites within a local bay management area must adhere to strict traffic pattern and disinfection guidelines. Finally, the Fish Health and Biosecurity Plan establishes specific fallowing procedures and plans. Length of fallowing and degree of site disinfection are determined by a specific site’s health status. Sites that have had any clinical disease outbreaks or any cages depopulated due to confirmed positive ISA<sub>v</sub> test results are required to fallow for longer and disinfect at higher levels.

### **Disinfection Protocols**

Each operation in a bay management area is required to develop a site-specific Disinfection Plan (DP), which has to be reviewed and approved by the Local Bay Management Group. The DP specifies materials, methods, procedures, and documentation required in all disinfection activities of personnel, operations, and product; addresses risk levels of all phases of cleaning, disinfection and isolation; and identifies specific operational circumstances where disinfection is mandatory. Wherever possible, site-specific DPs must address all current known transmission and infection risks and include procedures that assure that subcontractors understand and follow disinfection guidelines.

### **Underlying Assumptions**

The agreement and local plans are predicated on the assumption that disease risks and operational practices at one facility may affect other facilities in the same general area. Thus, a collective interest coupled with the legal obligation to work together to minimize disease risk gives the Bay Management Agreement its power. The speed and degree of development of local bay management plans will be one measure of the efficacy of the agreement as an administrative and policy entity. More importantly, the results of systematic, statewide biosecurity audits will measure whether the Agreement and local plans are affecting operational practices that increase disease risks. Finally in the event of clinical disease outbreaks, the speed and degree of collective response will also be a measure of the agreements success in developing improved cooperation and coordination.

### **USDA ISA Program**

The industry is committed to responsible ISA management and seeks supportive and complementary ISA policy development from both Federal and State regulators in order to foster optimal disease management. Other authors at this

symposium will give a more complete summary of the USDA ISA Program. My presentation will focus on the intent and implementation of the program from the industry perspective.

In 2000, based on the landed value of farmed finfish and shellfish, Maine was the largest marine aquaculture producer in the Nation. Farm gate sales for Atlantic salmon alone exceeded \$100 million. The Maine salmon farming industry accounts for more than 1,000 full-time jobs located in some of the poorest counties in the United States. ISA losses and indirect costs to the Maine industry exceeded \$20 million in 2001 and 2002.

Although the Maine salmon-farming sector is significant in the United States and Maine, it represents less than 2 percent of world production of farmed salmon. Maine salmon farmers work in a highly competitive world market with other producing areas that have significant government programs that support their salmon producers. This has been recognized by the International Trade Commission, which has twice awarded Maine salmon growers judgments finding that foreign producers enjoy significant competitive advantages due to government support.

The ability of the salmon-farming industry in Maine to effectively manage any disease is dependent on grower initiatives, effective detection and treatment methods, sound fish health regulatory policy, and government assistance. Government assistance should take three forms—the same forms that are typical of U.S. Government assistance for terrestrial farmers.

- Disease surveillance and monitoring, including pathogen surveys, biosecurity audits, and quality control of testing laboratories and detection methods;
- Epidemiologic analysis and research designed to develop farming methods that reduce disease risks; and
- The development and implementation of an adequately funded indemnification plan to compensate private farmers for the value of seized animals they are forced to depopulate under disease

control programs. Indemnification should also fully cover the costs of depopulation, cleaning, and disinfection.

Although the USDA program contains elements of these three important areas, the Maine salmon-farming industry is concerned about the continued commitment of USDA to domestic salmon growers. Indemnity is a key concept to encourage active, early, and sustained participation in an effective ISA management program. Commercial insurance policies of the type popular in Norway typically have not found favor with Maine salmon farmers. Where partial losses may be covered, large deductibles apply on a per-incident basis. Importantly, losses that are the result of the depopulation of cages, either electively or in compliance with regulatory agency mandates, are not covered.

The industry believes that an adequately funded and professionally administered indemnity program is critical in order to facilitate the implementation of ISA Best Management Plans being implemented by individual companies and the Bay Management Plans. These initiatives are detailed and aggressive attempts to improve operations and reduce the risk of disease.

The industry has spent and continues to spend significant capital to implement these initiatives. Current indemnification funding levels are substantially below actual industry costs. Future funding levels appear likely to be even lower than current levels.

Cooperation is needed between the private, public, and academic sectors to research, develop, and fund better indemnity strategies. Options such as State, Federal, and/or industry-sponsored indemnification should all be explored fully in order to increase the total funding available for indemnification. In particular, Maine State policy prioritizes the responsible development of marine aquaculture as a means of diversifying coastal economies (Maine Aquaculture Development Committee, 1990, Maine Department of Marine Resources and Maine Coastal Program 1997). Farmed salmon is currently the second most valuable

seafood product landed in Maine. As of September 2002, the State has made no direct financial contribution to indemnification.

## Where Do We Go From Here?

Four critical areas deserve more attention in order to improve the efficacy of ISA control and management in Maine:

- Significantly increased biosecurity auditing,
- Research on the epidemiology of ISA,
- Increased indemnification funding, and
- Increased cooperation between Canadian and United States growers and regulators.

Frequent and comprehensive third-party biosecurity audits provide direct feedback to farmers and quickly address sources of risk and improve operational practices. The results of biosecurity audits should be openly discussed in local bay management group meetings in order to cooperatively solve issues and use peer pressure and threats of peer litigation to improve operations on substandard farms.

Systematic epidemiologic research should be done to improve our understanding of disease vectors and risk factors. In order to be most effective, this research must be conducted in a manner that does not compromise veterinary–client confidentiality. Research results should be used to review and improve current bay management biosecurity and fish health protocols.

Current indemnification levels are not adequate. The industry’s position remains that mandatory depopulation orders constitute a seizure of private property and that, under Federal and State law, the government is required to compensate the owner of said property. The industry is committed, however, to examining the possibility and potential form of a self-insurance component in a cooperative indemnification program.

In New Brunswick, Canada, the fish-farming industry sought changes to existing insurance

regulations to form a self-managed fund. Currently, this fund receives annual contributions from individual farms, paid in at the rate of 3 cents per pound of overall production. This level is matched by government funding. The industry component alone is not enough to support the current level of claims against the fund. In Maine, a similar industry match would generate only \$780,000. Given the level of Maine farmers’ losses to ISA in 2001 (approximately \$20 million) and the proposed funding and compensation levels (40 to 60 percent of industry production costs) for indemnification in 2003, there appears to be a significant gap between available funds and industry costs. The MAA and its member growers stand ready to work cooperatively on this serious challenge.

Maine salmon farms in Cobscook Bay share essentially the same water resources as Canadian farms. In several instances, farms from the two countries are located within 2,000 feet of each other. Although governance structures are different, Canadian and U.S. fish farms and their respective practices directly affect each other. Coordination of ISA management programs in Canada and Maine is essential in order to reduce risks in both jurisdictions. The MAA has opened discussions with its sister producer organizations in New Brunswick and Nova Scotia to improve communication and harmonize bay management practices. Similar discussions are occurring between regulatory authorities in both jurisdictions. These discussions must be accelerated, and legal structures must be developed that facilitate this cross-border initiative.

USDA’s ISA program has been critical to the rapid and aggressive response to the emergence of clinical ISA in U.S. waters. Regional and field personnel from USDA’s Animal and Plant Health Inspection Service have provided invaluable assistance to Maine salmon farmers in a timely and professional manner. Headquarters staff and the Secretary of Agriculture have provided vital leadership and support for a new program. Continued close cooperation between Federal and State authorities and the growers will significantly

increase the effectiveness of any ISA virus control and discuss eradication efforts.

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The author thanks the members of the Maine Aquaculture Association Fish Health Technical Committee for their hard work addressing the challenges ISA has presented over the last several years. The committee has been assisted by a number of external fish health professionals. Two people in particular deserve recognition for their extensive work and patience. Dr. Michael Opitz was instrumental in developing the first biosecurity audits and forcing us as growers to face our own mistakes and weaknesses. Dr. Peter Merrill has worked tirelessly to help write the industry's ISA Action Plan and has continued the development and implementation of Dr. Opitz's biosecurity audits. I thank them both for their efforts, time, and dedication to the continuing the viability of Maine aquaculture.

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# Survey of Nonsalmonid Marine Fishes for Detection of Infectious Salmon Anemia Virus and Other Salmonid Pathogens

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**Abstract:** In an effort to identify potential reservoirs of salmonid pathogens, nearly 3,000 fish, including alewife (*Alosa pseudoharengus*), American eel (*Anguilla rostrata*), Atlantic herring (*Clupea harengus harengus*), Atlantic mackerel (*Scomber scombrus*), Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), Atlantic halibut (*Hippoglossus hippoglossus*), pollock (*Pollachius virens*), American shad (*Alosa sapidissima*), and winter flounder (*Pseudopleuronectes americanus*), were sampled from the natural environment. Pollock, cod, and lumpfish (*Cyclopterus lumpus*) also were sampled from within cages holding infectious salmon anemia (ISA)-diseased salmon. Assays included cell culture for listed salmonid viruses, the direct fluorescent antibody test for *Renibacterium salmoninarum*, and reverse-transcriptase–polymerase chain reaction (RT–PCR) for ISA virus (ISAv). All of the fish collected from the natural environment tested negative by any assay method. Two of 12 pollock taken from inside a cage with ISA-diseased salmon showed weak RT–PCR

positive results and were cell-culture negative. Ninety pollock collected outside a cage with diseased salmon tested negative for viruses and *R. salmoninarum*. One of 24 pools (5 fish per pool) of tissues from cod taken from a wellboat holding salmon from a cage with clinically diseased fish produced cytopathic effects (CPE) characteristic of ISAv on salmon head kidney cells. This finding was confirmed by RT–PCR of cell culture supernatant. Viral pathogens and *R. salmoninarum* were not detected in 26 lumpfish collected from inside diseased cages. These data suggest a need for attention to biosecurity practices concerning nonsalmonids retained in and harvested from salmon cages. These results indicate that pollock and cod can harbor ISAv; however, it was not determined if the virus can replicate within these hosts. The significance of such potential carriers to the epizootiology of ISA needs further investigation as a source of the virus in the wild and to examine potential impacts on nonsalmonid populations.

## Introduction

Infectious salmon anemia (ISA)—a viral disease caused by an orthomyxovirus, possibly a member of a new proposed genus, *Aquaorthomyxovirus* (Krossøy et al. 1999)—has resulted in serious impacts to the Atlantic salmon (*Salmo salar*) farming industry in several countries. The disease was first reported from Norway in 1984 (Thorud and Djupvik 1988). In more recent years, cases of the disease have been reported from eastern Canada (Mullins et al. 1998), Scotland (Rodger et al. 1998), the Faroe Islands (Office International des Epizooties [OIE], 2000), Chile (Kibenge et al. 2001), and the Northeastern United States (Bouchard et al. 2001).

Outbreaks of ISA in the United States have been confined to Cobscook Bay, Maine, a fishery that accounted for more than 50 percent of the 36 million pounds of Atlantic salmon culture produced in the State in 2000. Within 10 months of the first officially reported case of ISA in February 2001, fish in more than 90 percent of the culture sites in Cobscook Bay

were diseased. This situation caused the State of Maine Department of Marine Resources (DMR) and the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (USDA–APHIS) to order eradication of approximately 1.5 million ISAv-infected or -exposed fish. Government-mandated depopulation followed earlier removal by the industry of more than 1 million ISA-exposed fish in efforts to control the disease.

Understanding the epizootiology of the disease is important in its control; therefore, identification of potential reservoirs of the pathogen in the natural environment becomes key to development of disease control measures. In laboratory studies, brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) have been shown to be asymptomatic carriers of ISAv that can transmit the virus to salmon by cohabitation (Nylund and Jakobsen 1995, Nylund et al. 1995 and 1997). Results of recent studies conducted in Scotland and Canada indicate that ISAv exists at a low prevalence level in wild salmonids and that ISAv has been found in Atlantic salmon aquaculture escapees (Olivier 2002, Raynard et al. 2001).

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(NMFS–NEFSC) has initiated a broad survey to examine possible reservoirs of ISAv and other salmonid pathogens in wild marine fishes. Because Cobscook Bay of Maine has relatively low salmonid populations, NMFS scientists hypothesized that nonsalmonids potentially could be reservoirs and possibly play a role in transmission of the virus between culture facilities. Species that are common to coastal Maine and Atlantic salmon culture sites were targeted in this study. These fishes were collected adjacent to and distant from culture sites in Cobscook Bay.

Furthermore, in November 2000, Atlantic salmon in eight Maine rivers and streams (the Dennys, East Machias, Machias, Pleasant, Narraguagus, Ducktrap, and Sheepscot rivers and Cove Brook) were listed as endangered under the Endangered Species Act. Due to the close proximity of salmon cages to some of these rivers, there was concern about the potential threat of disease to these endangered salmon. In addition, transmission of ISAv from asymptomatic, infected trout to Atlantic salmon by cohabitation has been demonstrated (Nylund and Jakobsen 1995). This fact caused consideration of potentially infected nonsalmonids to affect migratory Atlantic salmon as well. Therefore, sampling included nonsalmonids collected from selected Atlantic salmon rivers in Maine.

## Materials and Methods

### **Fishes Sampled**

Data presented here are for nonsalmonid marine fishes collected during 2000–02 from locations adjacent to and distant from Atlantic salmon culture operations in Cobscook Bay. With the exception of fishes collected from within or near Atlantic salmon culture cages, all fish collections were made ancillary to field programs having other objectives. Fishes were collected from the natural environment and from Atlantic salmon culture cages. Fishes from the natural environment were collected

- (1) From the Dennys, Narraguagus, Pleasant, Sheepscot, and Penobscot rivers in Maine during springtime smolt trapping operations;
- (2) From coastal Maine during DMR trawl surveys including areas from Penobscot Bay, Maine, to the border with Canada;
- (3) From offshore areas in the Northwest Atlantic during NMFS bottom trawl surveys from New Jersey to the Gulf of Maine; and
- (4) By angler catches within 10 m of Atlantic salmon culture cages in Cobscook Bay, Maine.

Fishes from the caged environment were taken from inside cages or boat wells during harvest operations of ISA-diseased Atlantic salmon in Cobscook Bay.

Initially, fishes were frozen for ease of handling in the field. Subsequently, fishes were chilled, transported to the laboratory of Micro Technologies, Inc. (Richmond, ME), or to field stations for dissection, or were dissected fresh onboard survey ships. Tissues were appropriately preserved for the various assays (Thoesen 1994).

We aimed to sample each species at least at the 5-percent prevalence detection level (i.e.,  $N = 60$ ) and oftentimes were able to sample at the 2-percent prevalence detection level ( $N = 150$ ). Species sampled from the natural environment included alewife (*Alosa pseudoharengus*) ( $N = 1,059$ ), American eel (*Anguilla rostrata*) ( $N = 297$ ), Atlantic herring (*Clupea harengus harengus*) ( $N = 684$ ), Atlantic mackerel (*Scomber scombrus*) ( $N = 211$ ), Atlantic cod (*Gadus morhua*) ( $N = 115$ ), haddock (*Melanogrammus aeglefinus*) ( $N = 55$ ), Atlantic halibut (*Hippoglossus hippoglossus*) ( $N = 2$ ), pollock (*Pollachius virens*) ( $N = 123$ ), American shad (*Alosa sapidissima*) ( $N = 3$ ), and winter flounder (*Pseudopleuronectes americanus*) ( $N = 259$ ). Nonsalmonid species collected from inside Atlantic salmon culture cages included Atlantic cod ( $N = 120$ ), pollock ( $N = 16$ ), and lumpfish (*Cyclopterus lumpus*) ( $N = 26$ ). Table 1 summarizes the species and numbers of fishes tested and their general locations of capture. Figure 1 illustrates the general locations of fishes sampled from the entire survey area, and

figure 2 highlights sampling locations in Maine waters.

**Table 1—General locations, fishes, and number sampled for disease assays**

Location	Common name	N
<b>Natural environment</b>		
Rivers in Maine	Alewife	944
	American eel	297
Coastal Maine	Alewife	22
	Atlantic herring	230
	Atlantic cod	90
	Pollock	7
	Winter flounder	189
Ocean	Alewife	93
	Atlantic herring	454
	Atlantic mackerel	211
	Atlantic cod	25
	Haddock	55
	Atlantic halibut	2
	Pollock	26
	American shad	3
Winter flounder	10	
Near cages with ISA-diseased salmon	Pollock	90
	Winter flounder	60
Within cages holding diseased salmon	Atlantic cod	120
	Lumpfish	26
	Pollock	16

### Assay Procedures

The tissue sampling and assay protocols were performed according to “Suggested Procedures for the Detection of Certain Finfish and Shellfish Pathogens” (Thoesen 1994). Fishes in the study were sampled for the following assays: viral isolations, ISAv-specific RT-PCR, and direct fluorescent antibody test (DFAT) for *Renibacterium salmoninarum*, causative agent of bacterial kidney disease. Not all assays were conducted on all fish. Assay selection was dependent on the field situation (for example, NMFS-NEFSC bottom trawl surveys run 12 consecutive days at sea in an environment not conducive to cell culture).

**Viral Isolations**—Kidney, spleen, and gill tissues from a maximum of five fish per individual species were pooled, placed into phosphate-buffered saline (PBS), and kept chilled until further processed within 24 hours. Tissues were homogenized and diluted 1/10 in PBS and were further diluted 1/10 in Minimum Essential Medium (MEM) with Hank’s salts, L-glutamine, and 2-percent fetal bovine serum. Tissue homogenates were inoculated onto each of three cell lines: salmon head kidney (SHK), chinook salmon embryo (CHSE-214), and *Epithelioma papillosum cyprini* (EPC). All cultures were monitored for any cytopathic effect (CPE)-producing agents for 28 days at 16 °C. Suspected ISAv CPE was confirmed by ISAv/RT-PCR on cell culture supernatants.

**ISAv/RT-PCR**—Individual middle kidney tissue or blood were sampled from fishes and placed in RNALater™ (Ambion Inc., Austin, TX, USA). Samples were kept chilled at 4 °C and assayed within a few days or were kept frozen at -20 °C until assayed within a few weeks. RNA was extracted from kidney tissue or blood using the RNeasy® Mini Kit (Qiagen) and subsequently tested by an RT-PCR protocol using the ISAv 1D/2 primer set. Based on positive controls and appropriate sized marker bands, evaluators considered a resulting amplified band positive when it occurred at the 493 base pair migration position. RT-PCR products were purified and submitted for gene sequence analysis.

**BKD-DFAT**—Smears of kidney tissue from individual fishes were prepared, fixed in methanol, and refrigerated until processed. Fixed smears were stained with a fluorescent-labeled polyclonal *Renibacterium salmoninarum*-specific antiserum according to standard DFAT protocols. Stained slides were observed for bacterial fluorescence indicative of *R. salmoninarum*.

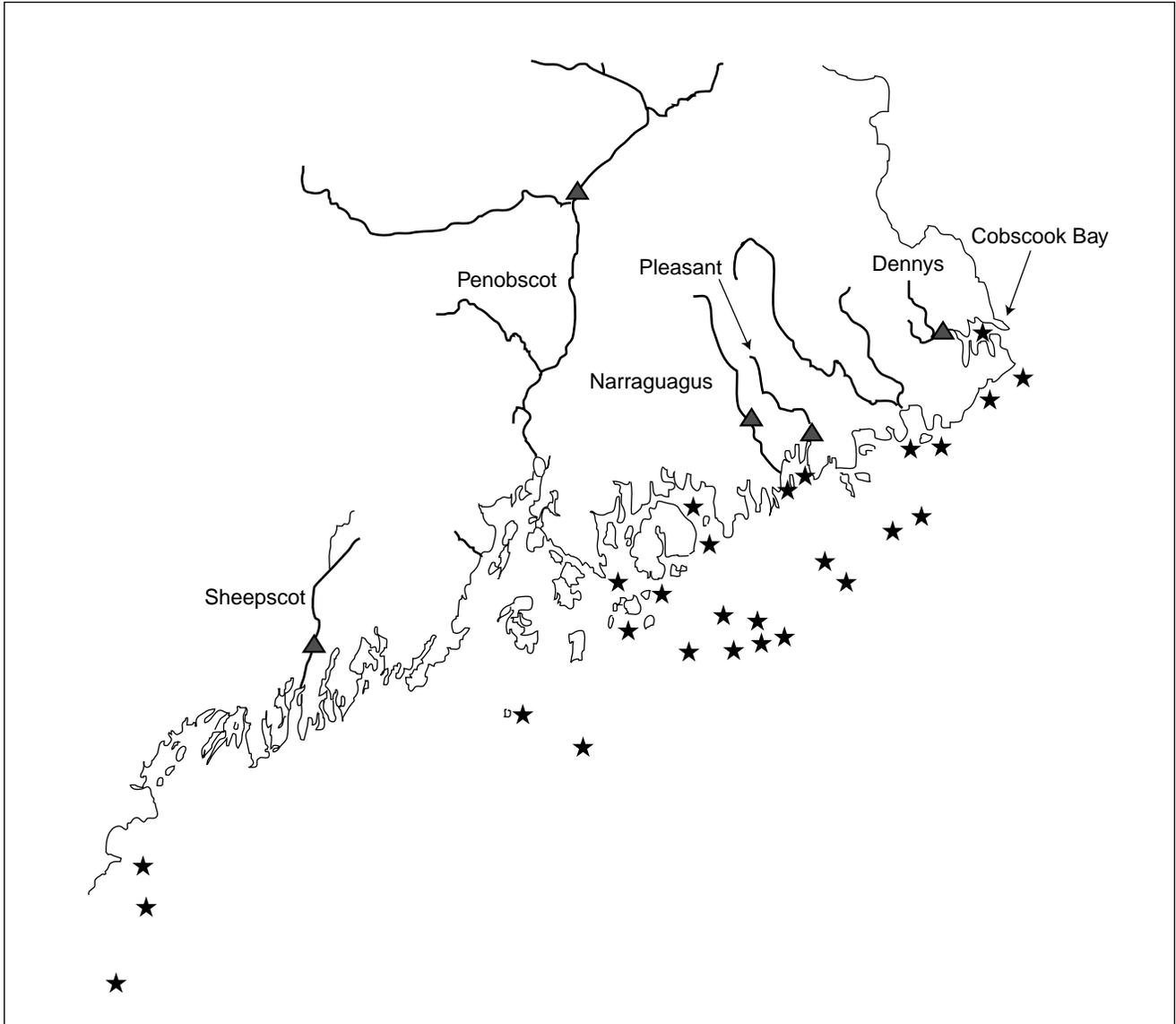
### Results

A total of 2,970 fishes, representing 11 species, were assayed for salmonid viral pathogens and *R. salmoninarum*, the causative agent of bacterial kidney disease (see table 1).

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**Figure 1**—Locations of fish collections, New Jersey to Maine, 2000–02.



**Figure 2**—Locations of fish collections in Maine, 2000–02.  
(Stars = coastal/ocean sites; triangles = river sites.)

### **Fishes From the Natural Environment**

Excluding Cobscook Bay, Maine, various species totaling 2,658 fish were collected from rivers and coastal areas in Maine and from ocean areas in Maine to as far south as New Jersey. Within Cobscook Bay, 150 fish were collected near salmon cages; 90 pollock were taken within 10 m of cages with ISA-diseased fish, and 60 winter flounder were collected from beneath cages holding uninfected salmon.

All fishes collected from the natural environment tested negative for *R. salmoninarum* by BKD–DFAT and for ISAv by RT–PCR. No CPE characteristic of ISAv or other salmonid viruses was observed in cell cultures of fishes from the wild.

### **Fishes From Cages With ISA-Diseased Salmon**

Fishes collected from cages of diseased Atlantic salmon came from two different sampling events. One event provided 12 pollock; the second event provided 120 cod, 26 lumpfish, and 4 pollock while cages were being harvested.

Two of 12 pollock taken from a cage with ISA-diseased salmon showed a weak positive band by RT–PCR for ISAv. CPE was not observed in corresponding cell-culture samples. Conclusive DNA sequencing results were not obtained from the PCR products due to the weak amplifications in these two pollock samples.

One of 24 pools (5 fish/pool) of tissues from cod taken from a wellboat containing salmon exhibiting clinical ISA yielded CPE characteristic of ISAv on SHK cells. RT–PCR assay of the cell culture supernatant confirmed the agent as ISAv. RT–PCR assays of the individual fishes that comprised the positive pool of tissues were negative for ISAv. Gene sequencing of the RT–PCR product confirmed that the CPE was due to ISAv and demonstrated 99-percent homology with the North American ISAv isolate. Assays on 26 lumpfish and 4 pollock collected along with the cod did not indicate the presence of viral pathogens.

No BKD–DFAT-positive results were detected among the several species of fishes taken from culture cages or the harvest boat.

## **Discussion**

The results of this study indicate that *R. salmoninarum*, ISAv, and other cultured fish viruses are not present at a significant level in the species of fishes tested from the natural environment. ISAv, however, was detected and isolated from nonsalmonid fishes that, as age-0 juveniles, became entrained in Atlantic salmon culture cages.

The isolation of ISAv from tissues of cod taken from the harvest boat well presents an interesting and perhaps significant finding. Viral culture can stand alone as a definitive test for ISAv as it indicates the presence of viable viral pathogen. Yet, it is noted that the corresponding direct-tissue RT–PCR samples from the individual fish comprising the positive pool tested negative repeatedly. These confounding results have possible explanations. Because the cod were taken from a wellboat containing ISAv-infected fish from a site where salmon displayed clinical ISA and gill lamellae were included in the tissue sample, exogenous viral particles could have adhered to the gill tissue as a result of a high viral load in the boat well and thereby be carried into the cell culture assay. That scenario would account for the negative RT–PCR of individual kidney tissues of the five fishes that made up the pool. However, if the viral load in the harvest well was substantially high, it would seem probable that more than 1 of the 30 pools of fishes (cod, lumpfish, and pollock) collected at that time would have tested positive.

It is also possible that the cod were infected with ISAv, but with a low viral load as might be expected in a carrier state. Under these circumstances, it is possible for cell culture to detect virus that is not detected by RT–PCR, depending on the tissue distribution of low numbers of virus. In other laboratory studies that we have conducted to compare various tissue sources and ISAv detection

methods, CPE has been observed occasionally in cell culture without positive RT–PCR bands for the same tissue taken from known infected fish (unpublished data). Similar results were obtained in a wild fish survey in which ISAv was isolated from five separate sea trout each of which tested negative by RT–PCR (Raynard et al. 2001).

Two of 12 pollock collected at 1 time from within a cage holding ISA-diseased salmon showed weak bands by ISAv/RT–PCR assay. Ninety pollock collected adjacent to a cage holding ISA-diseased salmon tested negative for viruses by viral culture and ISAv/RT–PCR. The RT–PCR reactions from the two pollock did not produce substantial quantities of purified nucleic acid; consequently, the PCR products did not result in good gene sequencing reactions. Although it is uncertain if those two fish actually were infected with ISAv, it is possible that the bands represent amplification of degraded ISAv genome or a very low number of ISAv particles. It is worth noting that ISAv was not detected by RT–PCR in saithe (*Pollachius virens*) at a minimum of 7 days after intraperitoneal injection with the Norwegian strain of the virus nor in saithe cohabiting with infected Atlantic salmon (Snow et al. 2002). Because the corresponding cell cultures for the two RT–PCR-positive pollock were negative, the gel bands were weak, and the gene sequencing reaction was poor, the significance of the positive RT–PCR results remains unclear.

These results indicate that cod and pollock can harbor ISAv; however, it is unknown if the virus can replicate within these hosts. The cod, which were collected in January, ranged from 14 to 26 cm (mean, 18.6 cm) total length. Based on growth rates for the Gulf of Maine, these cod were about a year old (Pentilla et al. 1989). It is unknown just how long the cod and pollock were within the cages, although based on published growth rates, mesh size of the nets on salmon cages, and assumed more rapid growth for entrapped fish, we may roughly estimate that these cod were in the cages for 8 months. Also unknown are the extent and duration of their exposure to ISAv. Regardless, the positive test

results from cod and pollock in cages containing ISA-diseased salmon is useful information for industry and should bring attention to biosecurity practices concerning the handling and disposition of nonsalmonids retained in or harvested from salmon cages. The significance of these potential carriers to the epizootiology of ISA remains to be investigated.

In autumn 2000 and spring 2001, fishes collected for assay were frozen for ease of handling in the field. Although ISAv is still infective after freezing at –20 °C (Thorud and Torgersen 1994, as reported by Nylund et al. 1995) and can be isolated from frozen tissue, we have found the rate of recovery from frozen tissue is slightly less than 50 percent (unpublished data). It is possible that the viral assays of the frozen fishes in this study may have been compromised. If ISAv indeed is present in these fishes at a 2- to 5-percent prevalence, levels at which we sampled, we would expect to detect it in assays on fresh tissues from subsequent years, but we did not. The BKD–DFAT results on frozen tissue are valid, and in none of the samples (fresh, frozen, natural, or retained) was there a positive result.

Studies in Scotland and Canada indicate that ISAv is present in wild salmonid populations, but ISAv was not detected in the wild nonsalmonids examined (Raynard et al. 2001, Olivier 2002). We did not assay wild salmonids from Maine for two reasons: wild Atlantic salmon in Maine rivers are an endangered species so the few salmon returning to rivers should not be put at risk, and populations of other native anadromous salmonids are small and scattered in Maine (Joan Trial, personal communication). We did test Atlantic salmon from the commercial West Greenland fishery and found 1 of 19 positive by RT–PCR (MacLean and Brown 2002). The gene sequencing of this PCR product confirmed ISAv and indicated the viral RNA was of the North American strain. Microsatellite DNA analysis to determine continent of origin of this fish indicated that it originated from North America (King et al. 2002).

Recent work by the U.S. Fish and Wildlife Service (USFWS) suggests that ISAv may be present in the wild salmonid population in Maine. When first

brought to the hatchery, 1 of 68 brood Atlantic salmon collected from the Penobscot River in Maine during 2001 tested positive for ISAV in blood using RT-PCR. Gene sequencing of this product indicated that the viral RNA was most similar to the Norwegian and Scottish strain of ISAV. Two subsequent tests over a 4-week period gave negative RT-PCR results, and the fish remained asymptomatic (Patricia Barbash, personal communication). Further assay of incoming brood fish showed no positive RT-PCR or viral assay results of 126 Atlantic salmon tested through August 2002. These fish will be tested again before spawning (Patricia Barbash, personal communication).

As the testing of wild salmon in Maine has been limited, it remains to be determined if a reservoir of the pathogen resides in salmonids and/or nonsalmonid species in the waters of Maine. Therefore, speculation on the source of the ISA virus in cultured Atlantic salmon in Maine cannot be made now.

## **Acknowledgments**

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# Infectious Salmon Anemia in New Brunswick: An Historical Perspective and Update on Control and Management Practices (1997–2002)

Sandi M. McGeachy and Mark J. Moore<sup>1</sup>

**Abstract:** Although infectious salmon anemia (ISA) has been present in New Brunswick since 1996, the causative agent was not identified until 1997. As a result, ISA spread from just a few farms to several farms in three bay management areas over a 2-year period (1996–97). A number of management and control procedures have helped reduce ISA viral loads and infection rates; these include single

year-class farming, early detection and removal of infected fish, fallowing, and containment of bloodwater and processing waste. However, ISA continues to be a serious disease of concern in New Brunswick. This manuscript discusses these management and control strategies as well as further measures that may be implemented to control ISA in New Brunswick.

## Introduction and History of ISA

The New Brunswick Atlantic salmon (*Salmo salar*) farming industry has grown from one farm in 1979 to 96 farms in 2002, and current production is estimated at 40,000 metric tons annually. All of these farms are located in southwestern New Brunswick, with close to 50 percent of the farms being within 30 km of each other. The industry has established 22 bay management areas (BMAs) within the southwestern part of the Bay of Fundy in an effort to facilitate single year-class farming and agreements on codes of practice.

In the summer of 1996, two sites in two BMAs (Lime Kiln Bay—BMA 10 and Bliss Harbour—BMA 9) experienced unexplained elevated levels of mortality among premarket size Atlantic salmon of the 1995-year class. Seventeen farms were located in these two bay areas within 5 km of each other (fig. 1). Disease investigations concentrated on bacterial pathogens before viral, toxicological, and histological assays were conducted. In the fall of 1996, additional farms in these bays also sustained elevated fish mortality. Various fish health specialists and services became involved in attempts to identify possible disease or toxicological agents associated with these losses. It was not until late in 1996, when common histological lesions were identified in the kidneys and livers of affected fish, that some form of a diagnostic test was used to describe the distribution of similar

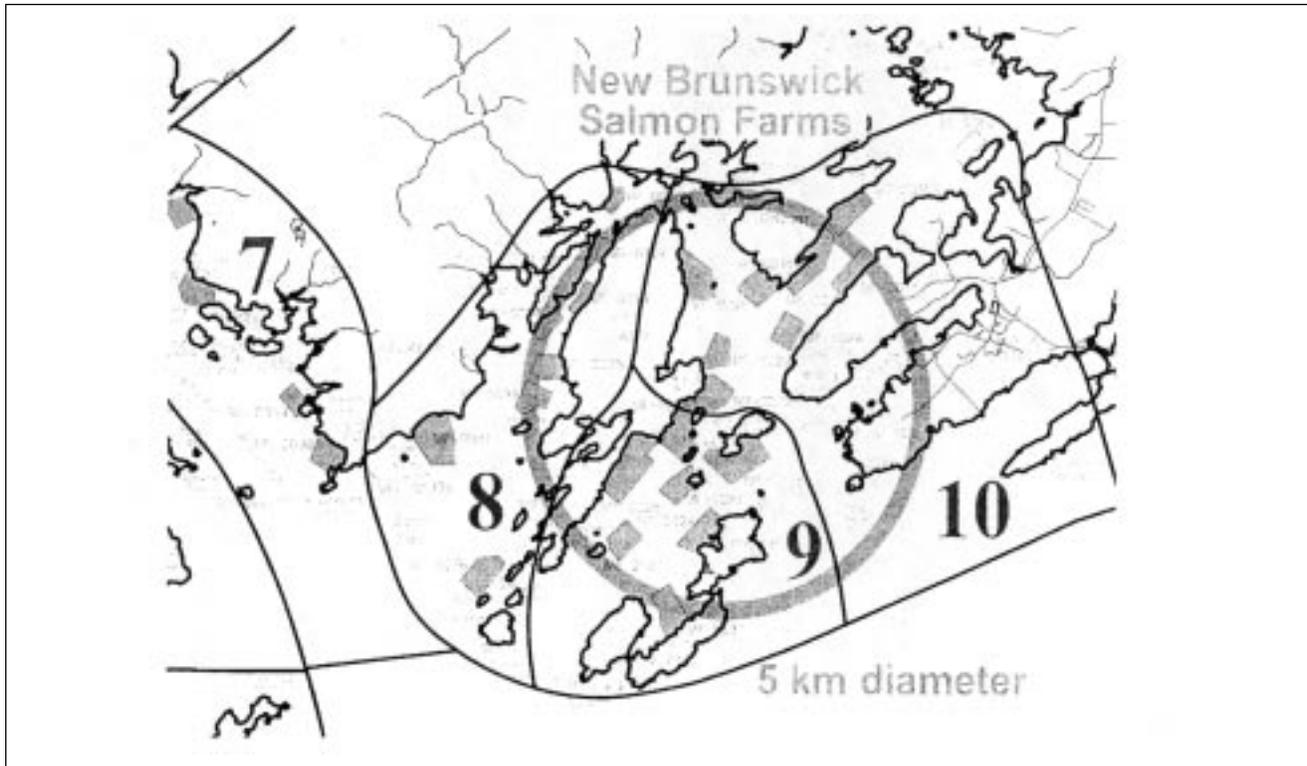
mortality events. At this time, the condition was termed hemorrhagic kidney syndrome (HKS) because the histopathology lesions were not consistent with those produced by any other known disease agents (Bryne et al. 1998).

Some researchers and companies also suspected that HKS was a new clinical presentation of bacterial kidney disease (BKD) due to the fact that fish at a number of farms with HKS problems had BKD. Managers of farms in these areas attempted treatments with various antibiotics but did not curtail HKS.

As part of disease testing and surveillance throughout late 1996, a togavirus was isolated in fish associated with HKS (Kibenge et al. 2000a). However, further transmission work under experimental conditions showed that this virus did not cause mortality in salmon, and the etiology of HKS remained unknown.

With consistent histological lesions being associated with the high levels of mortality, the New Brunswick Department of Fisheries and Aquaculture (NBDFA) completed an extensive histological sampling survey of fish from all of the farms in the industry between February and September 1997. The early focus of the screening was placed on the original affected BMAs (9, 10, and 20) and neighboring BMAs (6, 7, 8, and 19) in an attempt to ascertain the spread of HKS epidemiologically. A total of 4,723 kidney samples from 767 cages on 69 sites were tested by histological examination. There were 373 positive fish from 109 cages at 20 sites located in 5 BMAs. From the fish tested, the percent breakdown of positive samples by year-class was as follows: 7.3 percent among the spring 1995-year

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**Figure 1**—Map of aquaculture bay management areas (BMAs) and sites for Lime Kiln Bay (BMA 10), Bliss Harbour (BMA 9), and Back Bay (BMA 8) in New Brunswick, Canada.

class in 11 cages; 7.8 percent among the fall 1995-year class in 8 cages; 62.3 percent among the spring 1996-year class in 60 cages; 18.9 percent among the fall 1996 in 16 cages; and 3.2 percent among spring 1997-year class in 5 cages. Prevalence of HKS was 0.5 percent among broodstock and an additional 540 fish were labeled as "suspect." The survey also noted that 951 fish (18 percent) were found to have histological evidence of BKD infection, but only 2 of these fish were positive for HKS and 8 were considered suspect.

Because the cause of the mortality remained unknown, it was difficult to prevent the stocking of sites in affected areas (e.g., BMA 9, 10, and 20). Although a few farms did not place smolt on their sites in the spring of 1997, most farms did. The histological survey in 1997 had shown that HKS was contained within Lime Kiln Bay, Bliss Harbour, and

Seal Cove (BMA 20) located on Grand Manan Island. However, some survey work indicated that a limited number of fish tested positive for HKS in Back Bay (BMA 8) and Deer Island East (BMA 6), indicating potential spread into neighboring areas by the summer of 1997.

Further research and testing for a causative agent resulted in the isolation and identification of the infectious salmon anemia virus (ISAv) by the Research and Productivity Council (RPC) in September 1997. This virus was conclusively demonstrated to be the etiologic agent for HKS (Lovely et al. 1999), and further confirmation was provided by identification of ISAv samples sent to the National Veterinary Institute in Norway.

This was the first time ISA had been reported outside of Norway. This confirmation became a turning point that enabled the New Brunswick

government and industry to implement appropriate control and management strategies. A number of risk factors concurrent with ISA were already identified in Norway (Vågsholm et al 1994, Jarp and Karlsen 1997). Risks included

- Timing of detection and slaughter of infected fish and cages,
- Number of generations on a site,
- Proper handling and disposal of dead fish,
- A 5-km zone of influence for slaughterhouses and effluent discharges from processing plants near fish farms,
- Harvest procedures and bloodwater containment,
- Transportation corridors,
- Zoning of infected farms in high-risk “combat” zones,
- Fallowing of sites, and
- Disinfection protocols.

As a result of reviewing these risk factors and information contained in the action plan known as “Stop ISA—Norway 1994” (Anonymous 1994), new control and management strategies were implemented in New Brunswick (NB). The NB Integrated Management Plan for ISA was based on detection, containment, control, and prevention. The NB Department of Fisheries and Aquaculture (NB DFA) established an extensive ISA Surveillance Program. An Eradication Program, based on Ministerial-ordered depopulations of clinically infected fish, was also established.

The Government of Canada supported initial funding for the compensation program under the auspices of the National Disaster Relief Program. Industry and NB DFA also recognized the need for a comprehensive ISA Management and Control Program involving the adoption of stringent husbandry, harvesting, processing, and transport practices, including the disinfection of equipment and the replacement of wooden cages, wooden harvest barges, and wooden feed boats. A Market Protection Program was also developed by the industry, and a public relations firm was hired to prepare a

comprehensive plan that addressed issues related to human health and consumer perceptions.

Depopulation of infected stocks began with whole site removals in Lime Kiln Bay, Bliss Harbour, and Seal Cove. These areas were completely emptied of salmon from cages in June 1998 and then fallowed for 10 to 12 months prior to smolt placement in the spring of 1999. Work on disinfection procedures and harvesting guidelines were also being developed (Torgersen and Håstein 1995, Washburn and Gillis Associates, Ltd. 1998, Neill and Gunter, Ltd. 1999). Zones or areas of concern were implemented to some degree with mandatory harvesting of market-size fish and restriction on smolt placements in some areas (e.g., Back Bay—BMA 8).

During the spring of 1998, the New Brunswick government and industry established a Fish Health Technical Committee comprised of industry veterinarians and government representatives (both Federal and Provincial) to look at fish health issues for farmers in the Bay of Fundy and to provide advice to the New Brunswick Minister of Fisheries and Aquaculture. One such recommendation of the Committee was that smolt should not be placed into Back Bay (BMA 8) due to its proximity to Lime Kiln Bay and Bliss Harbour (within 2 km of affected farms). As it turned out, fish from three out of eight farms in Back Bay became infected within months of the smolt restriction order.

In 1998, a total of six additional farms with 1997-year class fish became infected with ISA as the disease spread to Back Bay (BMA 8), L’Etete (BMA 7), and Beaver Harbour (BMA 12). By this time, all of the farms in the three BMAs originally affected were fallowed, and it was apparent that total containment of ISA was not going to be achieved.

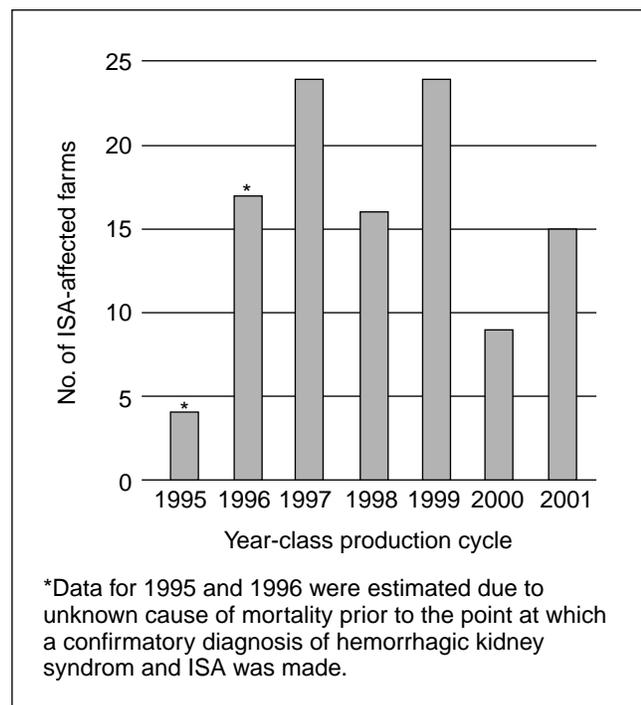
Total site depopulation was not an option at that time, but industry and government agreed that cage-by-cage removal of infected stocks was the best method of reducing the spread of ISA within and between sites. Further recommendations and Ministerial orders came for the early harvest of market fish (1997-year class) in Back Bay, L’Etete, and Beaver Harbour by December 31, 1998. By the

end of 1998, a total of 24 sites that had 1997-year class smolts were affected by ISA. This resulted in the depopulation of nearly 1.7 million salmon (table 1).

**Table 1—Atlantic salmon farms affected by infectious salmon anemia (ISA) in New Brunswick, Canada**

Year class	No. of ISA-affected farms	No. of salmon depopulated	Mean no. of salmon depopulated per farm
1995	4*	NA	NA
1996	17*	NA	NA
1997	24	1,667,870	69,495
1998	16	1,190,511	74,406
1999	24	1,636,518	68,188
2000	9	221,700	24,633
2001	15	985,000	65,667

\* Data for 1995 and 1996 were estimated due to unknown cause of mortality prior to the point at which a confirmatory diagnosis of hemorrhagic kidney syndrome and ISA was made.



**Figure 2**—number of Atlantic salmon farms affected by ISA in New Brunswick, Canada, by year-class production cycle.

Newly placed smolts of the 1998-year class became infected in Beaver Harbour in August (within 4 months of transfer to saltwater), while newly placed smolts in L'Etete became infected in the fall of 1998 (6–8 months after saltwater transfer). Sixteen sites with 1998-year class salmon became affected by ISA during their production cycle (fig. 2).

A 4-month fallow period was implemented for many of the BMAs affected by ISA. Fallowing was a difficult decision to make because scientific data did not exist on the minimum acceptable fallow period. On the west side of Deer Island, 9 out of 12 sites became affected (1998-year class), of which 6 sites were multiyear class (1998-year class and 1999-year class). As a result, newly placed smolts of the 1999-year class became infected within 4 months of transfer to saltwater. Once again, this infection scenario necessitated whole-site depopulations and harvesting of market fish to contain the spread of ISA.

By the end of the 1999-year class production cycle, ISA had affected close to 50 of New Brunswick's 86 farms, and more than 4,000,000 salmon had been depopulated. ISA had now spread beyond the inshore salmon sites in 1999 and was progressively moving down the west side of Deer Island. In total, 24 farms became affected by ISA in the 1999-year class (fig. 2). Over the next 2 years, ISA continued to spread slowly. Although the extent of the infection and losses were high, the losses were below the levels experienced with the 1997 to 1999-year classes (table 1).

## **Control and Management of ISA**

Some of the the main components of the NB Integrated Management Plan were modeled on information from Norway and based on advice from the Fish Health Technical Committee. One of the major components was the ISAv Surveillance Program which enabled the early detection and early removal of affected cages. Other components were based on restructuring the industry to single year-

class farming, compensation for ordered depopulated stocks, disinfection, controlling vectors such as sea lice, research, and containment of bloodwater and processing waste. All of the salmon processing plants completed infrastructure upgrades to allow for proper containment and treatment of processing waste.

## **ISAv Surveillance Program**

Once ISAv was identified as the causative agent for HKS (Lovely et al. 1999), industry and government established an ISAv Surveillance Program based on an extensive surveillance of the entire industry. Under guidance from the Fish Health Technical Committee, the ISAv Surveillance Program was based on early detection and removal of ISA-infected stocks on a cage-by-cage basis. Either government biologists/veterinarians or industry veterinarians visited sites showing no evidence of infection every 6–8 weeks, but suspect or positive sites were visited every 2–4 weeks. Weak or moribund fish were collected and necropsied at the NBDFA laboratory, where a full range of ISAv tests were completed (NBDFA 1998). The main objectives of the ISAv Surveillance Program were to detect the presence of subclinical ISA within cages and on sites at the earliest opportunity and to detect the early emergence of clinical outbreaks of ISA at the cage and site level.

Before the early 1990s, ISA diagnosis in Norway was based on evaluation of clinical signs, necropsy findings, histological changes, and hematological findings (Anonymous 1994, Håstein 1997). It was not until the mid-1990s that Norwegian diagnosticians used virus isolation (Dannevig et al. 1995) and experimented with indirect immunofluorescent antibody (IFAT) tests (Falk 1997, Falk et al. 1998) and reverse-transcriptase–polymerase chain reaction (RT–PCR) tests (DeVold et al. 2000). The IFAT and RT–PCR assays were not recognized by the Office International des Epizooties (OIE) as official or approved tests for ISA (OIE 1995 and 1997). In fact, the OIE (1997) clearly stated that

the diagnostic procedures for ISA were to be based on clinical, pathological, histopathological, and hematological changes. The IFAT test could be used to confirm the presence of viral antigens and to resolve cases that were otherwise inconclusive.

When the New Brunswick ISAv Surveillance Program was developed, it was decided to test each sampled fish for ISA by histological examination, virus isolation on SHK cell lines (Dannevig et al. 1995), IFAT, and RT–PCR. By 2000, histological tests were dropped as the reliability and repeatability of the IFAT and RT–PCR appeared to be sufficient for surveillance purposes. Currently, veterinary clinical impressions and IFAT tests on moribund fish samples are used for preliminary screening on all sites. Both RT–PCR and viral tissue culture are then used to confirm positive IFAT results. The ISAv Surveillance Program is in its sixth year, and an average of about 8,000 samples are analyzed annually.

## **Single Year-Class Farming**

As already mentioned, production of multiple year-class generations on a given farm is a key factor that increases the risk of ISA (Jarp and Karlsen 1997). It is estimated that in 1996, more than 60 percent of the salmon farms in New Brunswick were run as multiyear-class operations that had as many as three or four generations present on some sites. More and more farms have restructured to single-year sites and single year-class bays. Today, 98 percent of the farms are single year-class, with 100 percent of the farms completing this transition in the fall of 2002. However, it should be noted that single year-class farming, as currently defined by policy in New Brunswick, allows for up to 20 percent of the market fish to be held over on a site into their third year for a 4-month overlap with newly placed smolts (NBDAFA 2000). The holding over of market fish is contingent upon ISA status for that farm and farms in that particular BMA. Since it takes 14 to 16 months in seawater for salmon to reach market size, true single year-class sites would require the industry to maintain more than two sites to allow for full crop

rotation and complete following between year classes.

The incidence of ISA on multiyear-class sites for the 1999-year class of salmon, which became infected with ISA, was double the level on single-year class sites (table 2). Affected multiyear-class sites removed on average about six cages per site, as compared to three cages on affected single year-class sites (McGeachy 2001). Fish on multiyear-class sites also became infected within 6 months of smolt transfer, whereas infection did not occur until 12 to 14 months after transfer among single year-class sites.

A comparison of the data involving the number of affected farms and depopulation numbers was completed on four BMAs that had ISA in 1997–98. The bay areas of Lime Kiln Bay (BMA 10), Bliss Harbour (BMA 9), Back Bay (BMA 8), and Seal Cove

on Grand Manan (BMA 20) were the first to fully apply initial control and management plans for ISA by implementing single year-class sites and bays, disease surveillance, and containment of bloodwater and processing plant wastes. Comparison of disease statistics within these four bay areas indicated that the prevalence of ISA in the 1999-year class was reduced by 60 percent to 70 percent in comparison to the 1997-year class (table 3). In fact, ISA has not recurred in the Seal Cove BMA for two production cycles. With the exception of Bliss Harbour, losses and forced depopulations have been reduced from 900,000 salmon for the 1997-year class to just over 150,000 salmon in the 2001-year class. The prevalence in and losses of salmon to ISA in Bliss Harbour have not changed dramatically, which suggests that factors other than single year-class farming are important for controlling ISA and must be addressed to completely effect disease control. The mean number of salmon removed per affected site in Bliss Harbour has remained constant at about 100,000 fish per affected site. Current investigations are being conducted within this area to identify further control strategies. It should be noted that six previously affected BMAs have successfully gone through complete production cycles (18–24 months) and taken salmon to market without further reinfection.

**Table 2—Data on ISA at New Brunswick salmon farms comparing ISA-affected single year-class (SYC) and multiyear-class (MYC) sites**

Year-class	1998 SYC sites	1999 SYC sites	1999 MYC sites
Mean time (months) to ISA clinical disease	14	12	6
Mean no. of cages depopulated per affected farm	0	3	6

**Table 3—The history of ISA infection in four BMAs in New Brunswick, Canada, during three production cycles (1997, 1999, and 2001 year-classes)**

BMA	Farms	----- 1997 year-class -----			----- 1999 year-class -----			----- 2001 year-class -----		
		ISA farms	Fish removed	Mean fish removed per farm	ISA farms	Fish removed	Mean fish removed per farm	ISA farms	Fish removed	Mean fish removed per farm
Lime Kiln	10	9	583,881	64,875	2	25,577	12,788	3	95,000	31,667
Bliss Harbour	7	6	583,990	97,332	2	215,707	107,853	4	408,700	102,175
Back Bay	8	3	114,760	38,253	1	33,000	33,000	3	62,034	20,678
Seal Cove	4	3	206,823	68,941	0	0	0	0	0	0
Totals	29	21	1,489,454	70,926	5	274,284	54,865	10	565,734	56,573

## Compensation for Depopulation

Depopulation or stamping-out procedures have been considered a crucial component for the control of nontreatable viral infections such as ISA (Anonymous 1994). At the time when HKS and ISA was identified in New Brunswick, there was and still remains no national compensation program for the ordered depopulation of diseased fish such as those infected with ISA. As noted earlier, the Government of Canada jointly funded compensation with the Province of New Brunswick for a 3-year period. Not until 1999 were regulatory amendments completed to make it mandatory for farms to secure financing for compensation (self-compensation through industry). While depopulations were occurring (1998–2000), funds for compensation were always on a year-to-year basis, which was disconcerting to the farmers.

A long-term compensation program remains an issue with the industry and government. Under the auspices of the National Aquatic Animal Health Program (NAAHP), indemnification is being proposed to compensate farmers for cases such as ISA and other exotic diseases. The NAAHP not only includes a component on compensation but it will also develop guidelines and practices for disease surveillance, zoning, research, husbandry practices, and quality control/quality assurance in support of animal health for the aquaculture industry. This Program is still being developed jointly between the provinces/territories, industry, and the Federal Government of Canada.

## Research

After the industry was affected by ISA and the causative agent was identified, government and industry soon realized that a number of facts about ISA remained unknown. An HKS Science Committee of researchers, institutions and veterinarians was organized by government and industry to review research priorities and complete such research. Studies were completed on strain identification

(Kibenge et al 2000b, Ritchie et al 2001), vertical transmission of the virus from broodfish to offspring (Melville and Griffiths 1999), nonlethal detection methods (Griffiths and Mellville 2000), and epidemiology (Hammell and Dohoo 1999). Such research has filled scientific voids in basic knowledge concerning the disease process and remains an area of critical importance.

## Current and Future Initiatives

Current and future initiatives will focus on moving to true single year-class farming to allow for all-in, all-out production (complete fallow periods on all farms), improvements in biosecurity and husbandry, sustaining the industry from a fish health perspective, improving key infrastructures (e.g., wharves), and epidemiologic investigations to assist industry and government on improving upon current control and management practices for ISA.

## Conclusion

More than 55 percent of the salmon farms in New Brunswick have been adversely impacted by ISA at one time or another during the past five years (1997–2002). Although the disease has seriously affected the salmon farming industry, considerable progress has been made to control infection, reduce mortality and compensate for lost revenue. With the exception of a few BMAs, forced depopulation due to ISA has been reduced by as much as tenfold. Management factors that have been implemented to control the disease are at least partially responsible for the fact that ISA has not recurred in one bay area for three production cycles, and in another six bay areas, the disease has been absent for up to two production cycles. Thus it is promising to note that ISA can be effectively controlled and managed. Further improvements in management practices, fostered through advances in scientific research, will help industry and government authorities alleviate the deleterious biological and financial impacts of this disease.

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# Experiences With Regulatory Responses to Infectious Salmon Anemia in Norway

Kristin E. Thorud and Tore Håstein<sup>1</sup>

**Abstract:** Norway was the first country to experience the challenge of infectious salmon anemia (ISA). After the first outbreak in 1984, the number of cases of ISA exploded in 1989 and reached a peak of 80 new infected farms in 1990. The disease became notifiable in 1988, when scientists realized that ISA was contagious and putatively caused by a virus. Once the disease caused serious losses to fish farming, regulatory responses were developed in parallel with increased scientific knowledge. To eradicate ISA from infected farms and affected areas, control was mainly exercised through depopulation of the affected farm and eventually the surrounding area. To prevent introduction of

ISA into nonaffected areas, controlling transfers of live and dead salmon between areas has been effective. Within the last few years, there has been a resurgence in the number of outbreaks. There may be several reasons for this, none of which has been scientifically investigated. These include structural changes in the industry concerning transport of salmon for rearing or slaughter and enhancement of nonspecific yet protective immunity afforded by vaccination against other diseases. Although vaccination was not an alternative in 1990, it is currently considered to be a tool for the control of ISA within affected areas.

## History of ISA in Norway

Norway was the first country to experience the challenge of ISA. The first outbreak occurred in fall 1984 in a hatchery in Hordaland County in the southern part of Norway. Disease with similar clinical and pathological signs of infection occurred the next spring in farms that had received smolts from the originally affected hatchery. In northern Norway, the disease was first recognized in 1988, but one case might have occurred in 1986 that was not diagnosed.

Within salmon farms, the disease spread relatively slowly from one cage to another, and it generally took about 1 month for the disease to show up in fish in adjacent cages. Contagion of neighboring farms usually required about 6 to 9 months before clinical disease occurred. The first outbreak in an area usually occurred in a farm a couple of weeks after medication against another disease problem, such as sea lice, Hitra disease, or tapeworm infestations. If the first outbreak within a farm or area was initiated by some stress factor, it was difficult to stop the disease from spreading even after the specific stress was alleviated.

Although there were outbreaks without obvious origins, spread into new areas was initially seen after purchase of infected smolts or in farms located near slaughterhouses and processing plants. In Norway, and in Hordaland County particularly, the disease

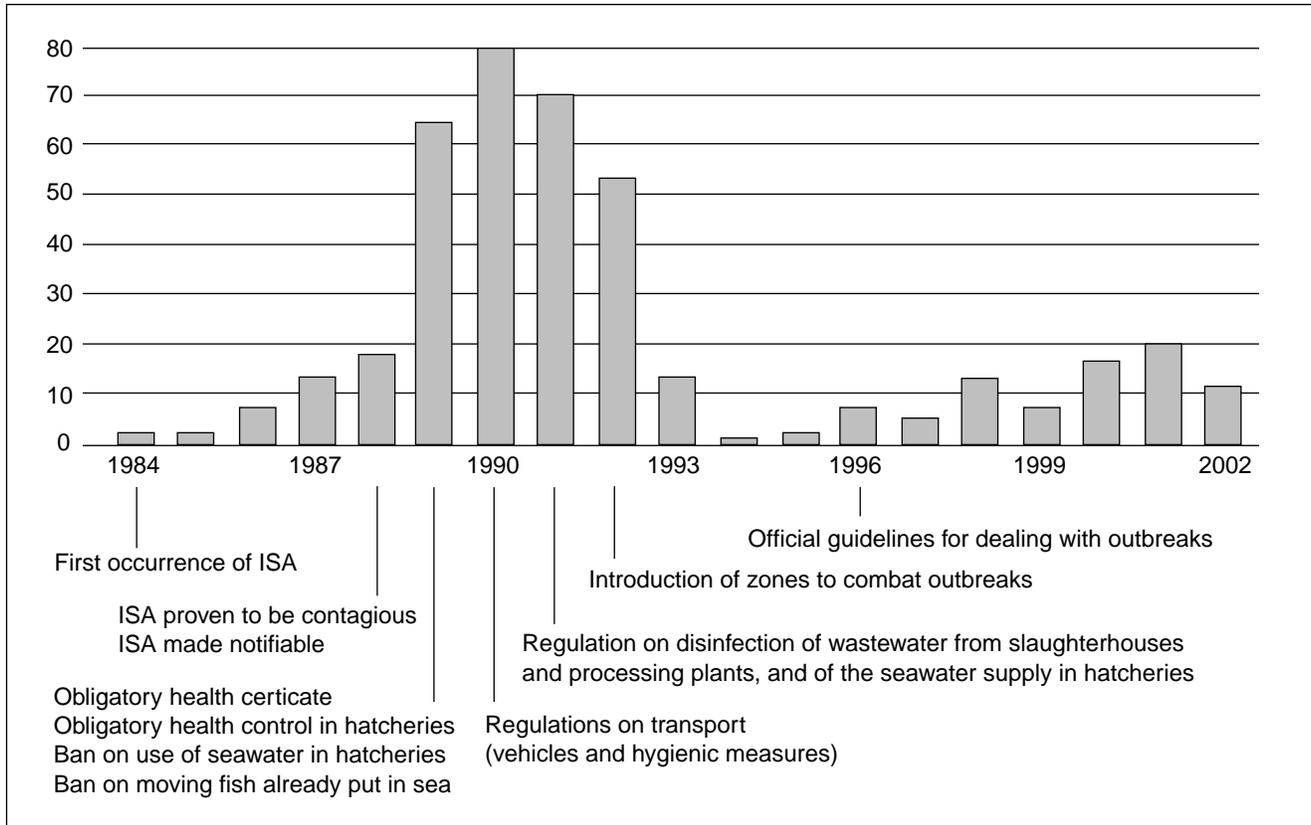
exploded in 1989 (fig. 1). It is interesting to notice that this happened the year after there had been massive towing of sea cages in all directions across the Hardangerfjord, which was done as an attempt to escape from a toxic algal bloom.

## Strategy for Controlling ISA

ISA became a notifiable disease within Norway in 1988, as a list B disease, when it was demonstrated that ISA was contagious (Thorud and Djupvik 1988). This gave the authorities the ability to introduce regulatory measures to control outbreaks. Still, it took many years before the virus was isolated (Dannevig et al. 1995) and preliminarily characterized (Mjaaland et al. 1997, Falk et al. 1997). In the interim, ISA caused serious losses in the fish farming industry, and the authorities had to develop a strategy to control it. This strategy included general measures adopted to prevent spread of virus and manage outbreaks at farms. The measures were based on knowledge of risk factors disclosed in field experiences, transmission trials, and epidemiologic studies. The strategy was developed in parallel with increasing scientific knowledge about the virus and risk factors for disease.

Two epidemiologic studies that were conducted in the first half of 1990s (Vågsholm et al. 1994, Jarp and Karlsen 1997) demonstrated that the risk for ISA was significantly related to the location of the farms and certain management factors. The risk for fish in a sea farm to contract ISA increased by 8.8 to as high as 13.9 when a farm was located closer than

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**Figure 1**—Verified outbreaks of infectious salmon anemia that occurred within Norway from 1984 until September 2002.

5 km from a location with ISA-infected fish. Risk increased by a factor of 14.6 when a farm was located closer than 5 km from slaughterhouses, and by 3.8 for processing plants that did not disinfect offal and wastewater. Risk from slaughterhouses was considerably reduced after disinfection of wastewater, but disinfection seemed ineffective on wastewater released from processing plants.

Management practices also increased the risk of contracting ISA on the farm. For example, the practice of sharing staffs with other farms increased the risk for ISA by 0.7 to 3.9, and removing dead fish less frequently than once a day increased the risk by 3.0. Mixing smolts from more than one hatchery slightly increased the risk for ISA by 1.6 to 2.9, but receipt of fish that were previously kept in other

seawater locations sometimes increased the risk for ISA by 56.2.

## **General Preventive Measures To Reduce Spread of ISA**

A connection was observed between the spread of ISA among fish farms, and particularly to new areas, with purchase of infected smolts and with release of untreated water into the sea from nearby slaughterhouses and processing plants. Regulations, therefore, targeted hygienic procedures at hatcheries, slaughterhouses, and processing plants.

To protect smolts produced in the hatcheries, it was considered important to ensure a pathogen-free water supply. Because ISA was only seen in fish kept

in seawater, disinfection of the seawater supply into hatcheries was introduced. This measure supplemented other general hygienic measures in the hatcheries, which required at least 12 regular health assessments per year. These controls facilitated early detection of disease, and also allowed the confident purchase of smolts from a particular hatchery. Disinfection of eggs is also part of these precautions.

To protect salmon in sea cages near slaughterhouses and processing plants, the authorities required that offal and wastewater from such facilities be properly managed and disinfected. Other measures included a ban on movement of fish after transfer to seawater and regulations for hygienic standards and procedures on transport vehicles, particularly wellboats. Demand for daily uptake of dead fish in summertime and every second day in winter was introduced in the regulations as a general precaution for fish disease control.

## Measures To Combat Outbreaks

From the very beginning of ISA's history in Norway, the disease tended not to disappear from affected farms until each site had been emptied of salmon and rainbow trout. About 1990, it became obvious that the disease could hardly be eradicated from the area until the authorities and the farmers agreed on a process to empty all farms and fallow the whole area for a designated period of time.

Based on these experiences and the information gained from epidemiologic studies, in 1996 the authorities introduced official guidelines to deal with outbreaks of ISA. Within the farms, all fish in sea cages had to be killed or slaughtered if their daily mortality exceeded 1 fish per 2,000 in the cage. Dead fish had to be removed daily and handled properly. The farmer's choice of slaughterhouse had to be approved by the local authorities, who closely reviewed the transport route for the fish to the slaughterhouse, as well as the hygienic standard practiced at the slaughterhouse.

After each affected farm was depopulated, it was cleaned, disinfected, and then fallowed for usually 6 months before any restocking was allowed at the site.

## Measures Taken Within the Zones

The official guidelines for dealing with outbreaks of ISA introduced both combat zones and observation zones around an affected farm. From the epidemiologic study demonstrating particular risk for sites located closer than 5 km from ISA-affected locations, a radius of about 5 km was used to establish the combat zone. According to the guidelines, managers were not allowed to put smolts into any site located within the combat zone. Additionally, a farm had to be fallowed for at least 1 month after it was depopulated and disinfected before permission could be given to restock the farm within the zone.

Intense official surveillance was conducted in all farms, giving priority to farms in the defined combat zone. Furthermore, official guidelines banned transports of smolts by wellboats closer than 20 km from infected sites, and transport of fish for slaughter had to pass more than 5 km away from ISA-affected farms.

## ISA Situation in Norway—2002

The number of outbreaks in Norway increased dramatically in 1989 and reached a peak of 80 newly affected farms in 1990. Since 1993, the number of outbreaks decreased, which suggested that the official control strategy was successful, even though current diagnostic tools were not available (fig. 1). Within the last 4 years, the number of outbreaks has increased again. Several reasons have been hypothesized for this reemergence, but none of these theories has been reviewed scientifically.

Certain areas have been subjected to more outbreaks than others. Still, the number of outbreaks

within the combat zones around the outbreaks has been few compared to the number of outbreaks occurring outside these zones (table 1). This fact indicated that the measures in force at affected farms have been relatively successful in preventing spread to neighboring farms, but the procedures to prevent emanation of the disease spread from affected areas have been less successful.

As a consequence of the introduction of regulatory measures connected to hygienic procedures of inlet water in hatcheries and wastewater in slaughterhouses and processing plants, the first outbreaks in new areas these days are rarely related to purchasing infected smolts or to being located close to slaughterhouses. Transports of smolts and fish for slaughter, though, have been suspected as a probable route of transmission for spread of ISA. Structural changes in the industry, leading to long transports of large amounts of salmon for rearing and for slaughter may be one factor. Unspecific immunization due to vaccination procedures against other diseases may to some extent have allowed spread of virus with undetected infected fish.

To reduce the risk for transports of infected fish through or to unaffected areas, or transports of uninfected smolts through affected areas, there is a need for early detection of infection. Early detection requires reliable diagnostic tools, but there must also be a stimulus for farmers to report suspicious findings. This is especially important because control of ISA is partly a question of confidence and cooperation between the authorities and the farmers. Still, strict regulations by the authorities are

necessary. The decade that has passed since ISA was a major problem for the industry gives the farmers today little experience in dealing with ISA and a lower level of awareness about necessary precautions.

## **Present Strategy**

Every year since 1984, Norway has dealt with different numbers of outbreaks of ISA. This scenario suggests that a control strategy allowing vaccination against the disease may be more practical in Norway than a total eradication strategy. Still, Norway has lessened the impact of ISA because regulations combined general preventive measures to reduce disease spread with an eradication strategy in and around affected farms. Structural changes in the industry and in management procedures have led to recognition of the need to enforce control in certain situations. Therefore, farmers and authorities in Norway have recently agreed to replace the guidelines for dealing with outbreaks of ISA with a contingency plan that in some respects goes even further than the regulations enacted by the European Union.

The aim of the contingency plan is to further reduce the risk for spread of virus from affected farms and areas to unaffected farms and areas. Strict regulations of aquaculture activities related to affected farms and the surrounding zones has been of utmost importance. At the farm level, the principal change in the new contingency plan requires depopulation of all fish within 80 days. Controls on transport of fish for slaughter and regulations on slaughterhouses will be strengthened. Furthermore, the contingency plan proposes a ban on all transport of live fish through combat zones and observation zones. Vaccination will be considered within the zones, when several outbreaks occur in the same area.

Early diagnosis of infected fish is important for success in the combat strategy against ISA. To enable farmers to detect ISA and to take necessary precautions to protect against it, the authorities will

**Table 1—Outbreaks of ISA in areas surrounding affected farms (= within combat zones) compared to outbreaks in unaffected areas (= outside combat zones)**

	1998	1999	2000	2001
Outbreaks within combat zones	2	2	2	6
Outbreaks outside combat zones	11	6	13	14
Verified outbreaks in total	13	8	17	20

plan a program to increase knowledge about signs of ISA, risk factors, and consequences of the disease among the farmers and the fish health services. Economic compensation to owners of ISA-affected farms for losses due to official restrictions after an outbreak of ISA would further encourage the farmers to report findings at an early stage. Such indemnification programs are under consideration by the Norwegian government.

## Conclusions

In summary, the Norwegian experiences with the regulatory responses in force support the principles for the strategy in force. Today's regulations emphasize general protective measures to prevent the spread of disease in combination with control strategies once outbreaks occur. Although vaccination was not a choice in 1990, it is considered within the framework of the new contingency plan as a tool to control ISA within heavily affected areas. A common vaccination strategy is not yet the choice.

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# Regulatory Aspects of Infectious Salmon Anemia Management in Scotland

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**Abstract:** Infectious salmon anemia (ISA) was first detected in a Scottish salmon farm in May 1998. Because ISA is a List I disease in European Union regulations, the outbreak was subject to immediate control measures by the Competent Authority in Scotland responsible for aquatic animal health measures. This included the removal of all stocks from the affected farm, the tracing of all contacts, the designation of infected and surveillance zones surrounding all foci of infection, and an epizootiologic investi-

gation into the spread and possible sources of the infection. Although ISA had spread to 10 additional farms, the control measures that were implemented successfully removed the disease from Scottish salmon farms within a year of the first outbreak, without any subsequent recurrence. The decision process for the management measures that were implemented was firmly based on principles of risk assessment.

## Introduction

Similar to other major salmon farming countries (Norway, Canada, the Faroes, Chile, the United States), Scotland has experienced an outbreak of infectious salmon anemia (ISA). In May 1998, the disease was first detected in one Atlantic salmon farm. Principally associated with farm management practices, the disease subsequently spread from that original source to another 10 farms that were widely distributed throughout the Scottish salmon farming industry.

Based on ISA experiences in other countries, particularly Norway, the risk of such an outbreak occurring in Scotland and more generally in the European Union (EU) had been previously recognized. Consequently, in the national regulations of both the EU and United Kingdom (U.K.), contingency measures already existed to manage such a situation. This paper describes the regulatory structures in place within Scotland to:

- (a) Require notification of the national Official Service for fish disease control of the occurrence of the disease,
- (b) Enable enactment of containment measures to restrict further spread,
- (c) Enable the introduction of management measures, and
- (d) Ensure that management measures were effective before relaxation of controls.

## Role of Regulations in Disease Management

In comparison with other approaches, regulatory intervention has a clearly defined but relatively limited role as a disease management tool. Most importantly, it can be directed only at restricting freedom of action of human activities that are significantly linked to a risk of disease occurrence. The objectives of regulatory controls are usually directed at a common or long-term benefit and often have a negative impact on an individual or restricted sector of the population. Realistically, regulatory intervention for significant diseases should be considered as an option only when other disease management measures are significantly less effective or are unavailable. Legislation is effective and credible only when it is based on scientifically validated information, when it can be practically implemented, and when its negative impacts are proportional to the benefits. Whenever there is noncompliance with a disease management regulation, these questions must be asked:

- Is there either a lack of conviction of the short- or longer term benefits, in which case an educational requirement is apparent, or
- Is the regulation wrong by not meeting the objectives, disproportionate in its effects, or inferior to another alternative, in which case the regulation may need to be changed?

As has been apparent from the ISA outbreak in Scotland, disease management regulations must have flexibility to accommodate new or unforeseen circumstances. Thus when some aspects of the EU regulations were believed to be impractical, changes

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were implemented in the immediacy of withdrawal, use of vaccination in disease containment, and provision of an option for compensation (Council Directive 2000/27/EC, Council Decision 2001/572/EC).

## **Role of Risk Assessment**

Countries that are members of the World Trade Organization (WTO) have rights and obligations with respect to trade, which are outlined as international standards in the Sanitary and Phytosanitary (SPS) Agreement of that organization. As the agency delegated by the WTO to deal with animal health, the Office International des Epizooties (OIE) provides details of these standards relevant to aquatic animals through the International Aquatic Animal Health Code. The Code provides guidelines that reduce the risk of spreading disease, while facilitating trade. Ethical standards and transparency are integral to the SPS Agreement, the OIE International Aquatic Animal Health Code, and national regulations.

When controls are imposed that implement regulatory disease management, the decision process demands scientific credibility based on risk assessment. The OIE International Aquatic Animal Health Code has now formally outlined the components of risk analysis (viz., hazard identification, consequences, risk of an incident, routine management measures available, and communication). These factors have long been recognized as an integral part of many fish disease management schemes that extend from farm husbandry practices to regulatory controls. This includes the U.K. Diseases of Fish Act 1937, 1983, which is one of the oldest and most comprehensive fish disease-management measures. Although this Act was developed to deal with the spread of furunculosis in wild salmonids within Britain, it proved highly adaptable for other disease risks associated with fish farming, including ISA.

It was, therefore, no surprise, when ISA was first detected in Scotland in May 1988, that the suite of actions taken by the Competent Authority closely

followed the OIE recommended pattern (McVicar 2001). Because data that supported a quantitative risk assessment approach on ISA management in Scotland were limited, considerable emphasis was initially placed on qualitative assessments of the disease in previously affected areas. Considerable cooperation was obtained from regulators and disease practitioners in both Norway and Canada in providing the latest information on the detection, diagnosis, and epizootiology of ISA in their respective countries. Such information proved to be invaluable in developing details of the management response to ISA in Scotland. However, they were treated with some caution because it could not be assumed that the disease would behave in the same way and present the same level of hazard in different affected areas. There were major differences in the local hydrography, ecosystems, and farming practices. Any of these could affect the transmission and effects of infection. Because such differences could also affect outbreaks in different regions within Scotland, each new incident of ISA or of suspected ISA in a Scottish salmon farm was subjected to a reevaluation of the risk assessment by an ad hoc group in the Competent Authority, taking into account all available relevant information. This response permitted the best use of the relevant local experience, which enabled a progressive move toward a quantitative approach in risk identification and management.

## **Hazard Identification and Notification**

ISA comfortably met the main criteria of a disease hazard suitable for control by regulations:

- It was of proven serious economic impact to farmed salmon;
- Currently, avoidance cannot be assured and available control measures do not prevent occurrence of serious disease; and
- It has a restricted distribution or occurrence within industries and geographically, a situation that can be maintained by restrictions.

These features are well documented for ISA in the scientific literature.

Early official awareness of the occurrence of incidents of a disease of concern, such as ISA, is a critical requirement that accompanies regulatory control. This is recognized by the OIE International Aquatic Animal Health Code, where a compulsory notifiable status is required for a disease to be included in a zonation system. ISA is included by OIE in the list of “other significant diseases.” In the United Kingdom, the risks from the disease were apparent from the situation in Norway during the 1980s; and, as a result, ISA was made a U.K.-notifiable disease in 1990, under the Diseases of Fish Acts 1937, 1983. Around the same time, the risks to salmon stocks throughout the EU were recognized and ISA was made an EU List I Disease in 1991, under Council Directive 91/67/EEC. Contingency plans to deal with suspected and confirmed outbreaks of ISA were specified in Council Directive 93/53/EEC.

These EU Directives were incorporated into U.K. national law by enabling legislation in the Fish Health Regulations 1992, Diseases of Fish (Control) Regulations 1994, and the Fish Health Regulations 1997. This suite of regulations provided the Competent Authority with the ability to establish and maintain Scotland as a zone free of ISA consistent with OIE recommended standards. With the outbreak of ISA in 1998, it was then possible to establish and maintain separation between infected zones, surveillance zones, and ISA-free zones by the same standards. Similarly, following clearance of the disease, steps were taken to reestablish the ISA free-zone status to Scotland.

## **Aspects of ISA Control Available Under U.K. Regulations**

Whenever EU Directives deal with an aspect of fish disease management by regulation, and enabling legislation is introduced into the U.K. as is required by the Directives, these new regulations take

precedence over previous official controls. Existing national power(s) remain where an area in existing regulations is not covered by the EU regulations.

The powers available to the Competent Authority of Scotland that were most critical to the successful management of the ISA outbreak include

- requirements for notification of suspicion or confirmation of infection in any susceptible species,
- introduction of disease containment measures on affected areas (infected farms, affected zones, and surrounding surveillance zones) with clearance (depopulation) of affected sites,
- provisions for an epizootiologic study to investigate the origin of the disease and its risk of transfer to other areas, and
- provision to require following of infected sites for a specified period before those sites can be restocked.

### **Early Warning**

For management of disease by regulation and to restrict its spread, it is essential that any evidence about the occurrence of the disease, even suspicion (both defined by the regulations), be brought to the attention of the Official Authority without delay. Internationally, it is a requirement of disease control through zonation, as in the OIE International Aquatic Animal Health Code, that a disease must be compulsorily notifiable. This principle is also embedded in both the United Kingdom and EU regulations. The requirement to notify the official authority in the U.K. is described as immediate, and failure to comply is treated as an offense. For professional diagnosticians, such an offense is likely to be considered as unethical. All susceptible fish species are covered by the regulations, an important point for emerging diseases because the full range of susceptible host species may be unknown.

### **Prevention of Spread**

Linked to immediate notification, it is self-evident that when an infectious disease is discovered or suspected in a location, the more rapid practical

disease containment measures are implemented, the better the opportunity to prevent spread. When ISA was detected in Scotland, it was possible to immediately place controls on affected facilities regarding movements of stock, equipment, persons, vehicles (including boats), and dead fish. Biosecurity provisions at entrances to the facility were also required. Similar restrictions were also placed on other farms in the designated infected (control) and surveillance zones surrounding the affected farm. Because ISA was a List I disease as defined in Council Directive 91/67/EEC (i.e., considered to be exotic to the EU), there was an obligation to implement an immediate compulsory program of depopulation. Depopulation involved immediate removal of all stocks from affected farms in a manner that managed the risk of further spread of the infection during processing or disposal of stocks. This proved difficult to achieve in large farms that produced several hundred tons of fish. Thus Council Directive 2000/27/EC amended this requirement to a more practical consideration in which all fish were withdrawn from the affected facility in accordance with a scheme established by the Competent Authority and approved by the Commission of the EU.

At the time of the outbreak, there was no provision in the EU or U.K. regulations for direct compensation for any of the stocks that were destroyed. The possibility of indemnification was later introduced through Council Decision 2001/572/EC.

After total depopulation, facilities and equipment were disinfected according to standards specified by the Competent Authority. As a possible additional tool for containment of infection, Council Directive 2000/27/EC lifted the total ban on the use of vaccines against ISA and permitted such immunization as a derogation within part of a contingency plan for an outbreak of ISA.

### ***Delineation of the Extent of the Disease***

Risk of spread of the infection from the detected source of the disease was evaluated through an

immediate census of stocks on site. Furthermore, an evaluation of official stock movement records maintained by each farm provided information on stock origins and shipments into and out of the affected facility. Through such an analysis it was possible to “ring fence” the ISA outbreak with respect to live fish movements by introducing further containment measures as necessary.

Information on possible sources of the disease and on the most important factors involved in the spread of the infection to other farms were obtained from the epizootic study, detailed results of which are provided in Alexander Murray’s article in this book. These studies provided the data on the most important areas where focus was required to support a transparent decisionmaking process, on both the management measures needed to control the outbreak and ways to deal with possible further risks of infection.

### ***Removal of Infection Foci***

To prevent recycling of ISA through different age groups of farm stocks, the Scottish regulations provided the authority to require the fallowing of an affected farm after an outbreak of the disease. However, it is a difficult task to validate a negative point biologically. Deciding to permit restocking of an affected farm without risk of recurrence of the infection is, therefore, difficult. Qualitative risk analysis on experiences elsewhere can provide information, but it could not be presumed that those procedures associated with ISA outbreaks in Norway and eastern Canada were fully relevant to the Scottish situation. Regulatory powers existed in Scotland to determine the period of fallowing before restocking a facility. As described in Ronald Stagg’s paper in this book, the absence of recurring infections in restocked farms after an outbreak on these indicated that a 6-month fallow period was sufficient to break any possible recurrence of the disease. Whether a shorter fallow period would be equally as effective is unknown. Based on an assessment of the infection risk (e.g., test results) and of the transmission risk (e.g., example the

proximity of other farms and traffic between farms), it was possible to allocate different minimum fallow periods in the control zones to farms where there was some suspicion of ISA infection (as defined by the regulations) and where there was no evidence of infection.

## Conclusions

Strong regulations to manage an outbreak of ISA existed in Scotland, and it was likely that the early implementation of these regulations had largely contributed to the elimination of this disease. Eradication of ISA from the Scottish salmon farming industry was accomplished within one year of the first outbreak as described in Stagg's chapter.

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# Design and Implementation of an Infectious Salmon Anemia Program

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**Abstract:** Late in 2001, the Secretary of Agriculture announced an emergency threat to the U.S. livestock industry and authorized the transfer of funds from the Commodity Credit Corporation to establish an infectious salmon anemia (ISA) program. Effective December 13, 2001, about \$8.3 million was authorized for the U.S. Department of Agriculture (USDA)–Animal and Plant Health Inspection Service’s (APHIS) Veterinary Services (VS) to implement an ISA control and indemnity program for farm-raised salmon in the United States. In addition to the payment of indemnity, these funds were designated to assist the State of Maine with program activities such as depopulation and disposal, cleanup and disinfection, establishment of surveillance programs, epidemiologic and diagnostic support, and training for producers and veterinarians. Maine officials have taken steps to prevent the

spread of ISA. However, Federal assistance was deemed necessary to control this threat to animal health and the U.S. economy effectively. APHIS’ goal is to control and contain the ISA virus through rapid detection and depopulation of salmon that have been infected, with or exposed to, ISA virus (ISAv). ISAv can be controlled within high-risk zones through surveillance, vaccination, and effective management practices. ISA control requires depopulation of all pens holding infected fish. Indemnification is necessary to provide an incentive for salmon farmers to report diseased fish and to continue testing.

Therefore, State officials asked USDA to assist with the epidemiology, surveillance, and indemnification programs. This report will explain the events that led to this emergency declaration and the current results of the newly instituted ISA indemnity and control program.

## Introduction: APHIS’ Involvement in Aquaculture Before ISA

During the late 1990s, APHIS received nearly two dozen petitions to promulgate animal health regulations and regulatory programs for the control of farm-raised fin fish as livestock. These petitions came from State farm bureaus, industry associations, individual producers, State officials, and businesses that serve aquaculture industries. These petitions covered dozens of issues and raised hundreds of questions. Although these requests were broad in scope, there was one underlying message: that aquatic farmers should receive the same services that domestic producers of livestock receive for animals moving in interstate and foreign commerce.

APHIS is authorized to promulgate regulations to protect the health of livestock and poultry in the United States and manages regulatory programs covering poultry, horses, swine, cattle, and other livestock. Additionally, APHIS programs cover animals that could transmit diseases or pests to livestock. Programs for terrestrial livestock are intended to (1) prevent the importation of diseases and pests, (2) regulate interstate movement in a uniform manner, (3) provide diagnostic laboratory services, (4) regulate vaccines and biologic reagents

used in animals, and (5) control or eradicate diseases and pests already found in the United States. Moreover, under the 2002 farm bill, the Animal Health Protection Act of 2002, APHIS has been granted the specific authority to promulgate regulations to protect the health of farm-raised aquatic animals.

In response to the petitions received, APHIS considered expanding its services and programs to include farm-raised finfish. Several services were already being provided to the aquaculture industry, including laboratory diagnostic work, endorsement of certificates of inspection for export for aquatic animals and aquatic animal products, and licensure of vaccines and biologic reagents for use in aquatic animals. The agency also controls damage by wild birds and other animals to farmed aquatic animals. User fees and cooperative agreements with State and local governments and industry pay for some of these programs and service, but expanding the agency’s services and programs would require additional funding.

To assess the specific needs of the aquaculture industry and to determine what role, if any, APHIS should play in regulating this industry, the agency issued an advance notice of proposed rulemaking in the *Federal Register* (64 F.R. 23795–23796) on May 4, 1999, entitled “Aquaculture: Farm-Raised Fin Fish.” In that notice, APHIS requested views and recommendations from all interested persons on several specific issues. First, should the agency

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consider regulating only domesticated farm-raised finfish or should it also consider regulating other aquatic animals? Further, what additional species, if any, should APHIS include in a potentially broader program? Second, if APHIS expanded the range of its services, what new or additional services should be provided? Additionally, should APHIS consider adopting specific regulations to prevent the introduction of diseases and pests of aquatic animal species, and if so, should those regulations be similar to those already in place for poultry and terrestrial livestock? Also, what form would any potential rulemaking take with regard to industry and State cooperative programs? Finally, should APHIS adopt regulations to control the interstate movement of aquatic species to prevent the interstate spread of diseases and pests? If so, should the agency consider including voluntary, industry-driven programs to help producers control and eliminate disease?

APHIS received 55 written comments in response to the advance notice of proposed rulemaking. Additionally, to facilitate public participation in the process, APHIS held a series of eight public meetings throughout the country in 2001. They were well attended and brought together a crosssection of industry representatives, State and local officials, and other interested parties. Meeting transcripts are available on the Internet at <http://www.aphis.usda.gov/ppd/rad/aquaculture.html>.

Currently, APHIS is evaluating comments received in response to the advance notice of proposed rulemaking and the comments we received at the public meetings. Once comment review is complete, APHIS will publish a notice in the *Federal Register*. Certainly, however, the agency's ISA control efforts indicate that preventing the establishment of foreign animal diseases in aquatic species is a vital priority within its mission to protect U.S. agriculture.

## APHIS and Foreign Animal Diseases

APHIS has regulations in part 53 of title 9 of the Code of Federal Regulations that provide for the control and eradication of diseases, including foot-and-mouth disease, rinderpest, contagious pleuropneumonia, exotic Newcastle disease, highly pathogenic avian influenza, or other communicable diseases of livestock or poultry that, in the opinion of the Secretary of Agriculture, constitute an emergency and threaten the livestock or poultry of the United States. The regulations authorize payments for the fair market value of the animals destroyed as well as payments for the cost of their destruction and disposal. The regulations also authorize payments for materials that must be cleaned and disinfected or destroyed because of disease exposure.

Although these diseases are included in the regulations because they constitute an emergency and threaten the livestock or poultry of the United States, they also pose a significant risk to countries that rely on the international trade of animals and animal products. To better facilitate international trade of animals and animal products, the United States is a member country represented at the Office International des Epizooties (OIE, a world animal health organization). OIE is an international veterinary organization comprising at press time (April 2003) 162 member countries in which APHIS—VS serves as delegate and Chief Veterinary Officer for the United States. Through this relationship, APHIS has mandates under the Agreement on Sanitary and Phytosanitary Measures (SPS Agreement) of the World Trade Organization (WTO) and, among other standards, disease reporting requirements to OIE. Although ISA is classified by OIE as an “other significant disease” it requires reporting within 24 hours because, in the United States, it is a new finding with the likelihood of exceptional epidemiologic significance.

## **The Emergence of ISA— Timeline of APHIS' Action**

The first case of ISA in the United States was confirmed in Maine on February 15, 2001. By December 2001, 19 cases of ISA had been confirmed in 16 net-pen sites in Maine.

On April 24, 2001, the Maine Department of Marine Resources, the Maine Aquaculture Association, and the Maine State Veterinarian requested that USDA provide the State with assistance in the areas of indemnification, epidemiology, and surveillance for ISA. Subsequently, APHIS entered into a cooperative ISA control program with Maine to help safeguard the salmon industry from future incursions of this exotic disease and to monitor and manage the ISA status of salmonid aquaculture sites in that State.

Effective December 13, 2001, and published in the *Federal Register* on December 20, 2001 (66 F.R. 65679–65680, Docket No. 01–082–1), the Secretary declared an emergency because of ISA. The emergency declaration process has several levels of clearances. The time between the submission for emergency status and the actual declaration of the emergency for a new disease in a new animal species can be lengthy.

The declaration of emergency was issued for many reasons. The declaration considered ISA's severe economic threat, not only to Maine's industry but also to the viability and sustainability of salmon aquaculture in the entire United States. Salmon production in Maine exceeds 36.2 million pounds annually, with an industry value of \$101 million (Maine Aquaculture Association 2001). In an attempt to control the ISA outbreak, by the time of the Secretary's declaration of emergency, the State's salmonid industry had already voluntarily depopulated about 900,000 salmon worth nearly \$11 million. This loss is even greater when capital expenditures such as labor costs and equipment are considered.

Additionally, the existence of ISA in Maine has affected other States well outside the threat of immediate disease transfer owing to its ramifications for international trade. As a party to the WTO, the United States must follow SPS Agreements. For example, when ISA emerged in Maine, Chile and the European Union prohibited the importation of trout and salmon eggs from Washington, Maine, Idaho, and any other State that would export them. The resulting trade loss of salmonid eggs for the year 2001 was estimated at \$2 million.

As a result of the Secretary's declaration of emergency, about \$8.3 million was transferred from the Commodity Credit Corporation for the Department's ISA control and indemnification efforts for fiscal year 2002, which ended September 30, 2002. ISA Funding for fiscal year 2003 would have to be requested through the Office of Management and Budget.

## **The Interim Rule—The Control Program and Indemnity**

After the declaration of emergency, APHIS began work to create a successful ISA control program that would gain the support of salmon producers. On April 11, 2002, APHIS published an interim rule (67 FR 17605–17611, Docket No. 01–126–1) that was effective retroactively to the Secretary's declaration of emergency dated December 13, 2001. The interim rule provided for, among other things, indemnification for fish depopulated because of ISA on or after the December 13, 2001, declaration of emergency. Since then, slightly more than \$6.1 million has been spent on indemnity, cleaning, and disinfection.

Additionally, the interim rule amended the definition of "disease," which is found in section 53.1 of title 9 of the Code of Federal Regulations, to include ISA among the other listed foreign animal diseases. Additionally, APHIS added other definitions to that section that help explain program elements. For example, a definition for the term "ISA Program Veterinarian" was added to identify the veterinarian as the person assigned to be the main point of

contact at the field level to manage the ISA program for APHIS in Maine.

The interim rule also provided requirements for eligible producers to receive indemnity funds, which are described in more detail next.

Finally, the interim rule evaluated potential economic consequences of the ISA control program in Maine. Because the entire farmed Atlantic salmon industry in Maine is at risk if ISA is not controlled, APHIS determined that the \$100-million-dollar-per-year industry outweighed the cost of the program. Specifically, APHIS anticipated potential benefits to the control and indemnity program. First, indemnity provides producers who have not been participating in ISA control with an incentive to do so. Additionally, APHIS recognized that a more aggressive approach early on, although the number of affected sites was relatively small, might obviate the need for higher future Federal costs to contain a more widespread outbreak. Additionally, the agency expected that the control and indemnity program might also reduce the impact of trade restrictions due to ISA. This reduction could produce third-party trade benefits by demonstrating to trading partners the intent and ability of the United States to protect its animal industries, thus enhancing U.S. ability to negotiate access to foreign markets.

## **Infectious Salmon Anemia Program Standards**

The ISA program standards establish procedures for the prevention and containment of ISA from farm-raised Atlantic salmon. These standards, which were codeveloped by the ISA Technical Board and approved by APHIS–VS, Maine’s Department of Marine Resources, and the Maine Aquaculture Association, provide industry, accredited veterinarians, approved laboratory personnel, and regulators with a template to follow to meet the goals of this Federal program. Indemnity payments, an integral part of this program, will be made to producers only if the established procedures and standards are followed. However, as described in the

interim rule, these indemnity funds are provided only for a 2-year period, during which time the industry should establish and implement a self-compensation indemnity fund based on a certain financial amount set aside per fish produced. Further, these standards are amendable as science and circumstances evolve.

### **Program Requirements**

To receive payment for ISA indemnity claims, producers must at a minimum observe the following requirements:

1. Establish and maintain a veterinary client–patient relationship with an APHIS-accredited veterinarian and inform the ISA Program Veterinarian in writing of the name of their accredited veterinarian at the time the participant enrolls in the ISA program and within 15 days of any change in accredited veterinarians.

The farmed salmon industry uses highly qualified personnel experienced in all aspects of fish culture, husbandry, and health management. Although most industry members have established inhouse procedures for increased disease surveillance and a working relationship with aquaculture veterinarians and diagnostic laboratories to provide further technical expertise, this requirement will ensure that all participants have ready access to an APHIS-accredited veterinarian, who will conduct the surveillance, testing, and reporting activities discussed in the next paragraph and will assist participants in carrying out the other aspects of the program discussed later.

2. Cooperate with and assist in periodic onsite disease surveillance, testing, and reporting activities for ISA, which will be conducted by the producers’ APHIS accredited veterinarian or a State or Federal official as directed by the ISA Program Veterinarian.

Surveillance ensures that resources and the attention of producers will be directed at routine and regularly scheduled inspections and health assessments of fish so that ISA will be quickly diagnosed. Testing with the best and most scientifically sound assays at an approved laboratory will ensure prompt and accurate diagnosis.

Reporting procedures ensure that, once infected or diseased fish are identified, control measures and depopulation can proceed rapidly.

3. Develop and implement biosecurity protocols for use at all participant-leased finfish sites and participant-operated vessels engaged in aquaculture operations throughout Maine. A copy of these protocols shall be submitted to the ISA Program Veterinarian at the time the participant enrolls in the ISA program and within 15 days of any change in the protocols.

The implementation of effective biosecurity protocols will reduce the risk of introducing and spreading ISA into and between marine sites and cages by movement of farmed fish, equipment, and people.

4. Develop, with the involvement of the participant's accredited veterinarian and the fish site health manager, a site-specific ISA action plan for the control and management of ISA. A copy of the action plan shall be submitted to APHIS for review at the time the participant enrolls in the ISA program and within 15 days of any change in the action plan.

The action plan is a document developed for each site that defines the response contingencies for ISA e.g., activities to be undertaken upon disease detection, notification procedures, etc.) should the disease emerge at the site.

5. Participate in the State of Maine's integrated pest management (IPM) program for the control of sea lice on salmonids. A copy of the management plan developed by the participant for the State IPM program shall be submitted to APHIS for review at the time the participant enrolls in the ISA program and within 15 days of any change in the management plan.

Sea lice are copepod arthropods belonging to the genera *Lepeophtheirus* and *Caligus*. Species of both genera infest Atlantic salmon and live in the mucus layer, where they attach and suck blood or cause sores. The larger *Lepeophtheirus* species are generally regarded as capable of transmitting ISA. Sea lice of both genera can cause stress on fish,

which adversely affects the immune response. The Maine IPM program for sea lice provides for monitoring, treatment, and management practices designed to minimize the presence of sea lice in pen sites and reduce the need for the use of chemicals and medications. APHIS considers control of sea lice to be a vital component of the ISA control program in Maine; therefore, the agency will, in cooperation with the State of Maine, review and verify the adequacy of each participant's sea lice management plan.

6. Submit to the ISA Program Veterinarian at the time the participant enrolls in the ISA program complete and current fish inventory information for each participant-leased finfish site with site and cage identifiers. Fish inventory information must include the numbers, age, date of saltwater transfer, vaccination status, and previous therapeutic history for all fish in each participant-leased finfish site.

This information will provide APHIS with the data necessary to establish disease control actions, complete epidemiologic assessments, and increase its ability to monitor fish populations effectively.

7. Maintain, and make available to the ISA Program Veterinarian upon request, mortality data for each participant-leased finfish site and pen in production.

This can be accomplished using existing industry records systems and log sheets. The mortality data will be used by APHIS in conjunction with the fish inventory information discussed previously to establish disease control actions, complete epidemiologic assessments, and increase the agency's ability to monitor fish populations effectively.

8. Cooperate with and assist APHIS in the completion of biosecurity audits at all participant-leased finfish sites and participant-operated vessels involved in salmonid aquaculture.

These audits will be performed to assess the efficacy of the biosecurity protocols established by the participants to reduce the risk of introducing and spreading ISA into and between marine sites and cages by movement of farmed fish, equipment, and people.

## Program Administration

**ISA Technical Board**—The ISA Technical Board consists of seven individuals: the USDA–APHIS–ISA Program Veterinarian, two Maine Department of Marine Resources (DMR) representatives, three industry representatives appointed by the Maine Aquaculture Association, and a chairperson. The board makes recommendations to the USDA–APHIS–Area Veterinarian-in-Charge (AVIC), ISA Project Manager, or the Maine DMR Commissioner, or all three, to consider positive laboratory results, epidemiologic data, audit reports, or other information regarding reported disease risks or conditions requiring action under the terms of this program. A quorum will be four people (at least two industry and two regulatory agency representatives) for any given meeting or conference call. The chairperson will serve as facilitator and vote only in the case of a tie.

### ISA Technical Board Committee Members

Chair: Michael Opitz, University of Maine	
Stephen Ellis	USDA–APHIS–VS
Paul Waterstrat	Maine DMR
Andrew Fisk	Maine DMR
Nils Steine	Atlantic Salmon of Maine
Dan MacPhee	Maritime Veterinary Services
Julia Mullens	Heritage Salmon

## Program Progress—The Science of ISA Control

**Surveillance Diagnostic Sampling and Testing**—As provided in the Program Standards, participating producers must submit to diagnostic testing procedures. Currently, surveillance sampling is conducted by APHIS on a monthly basis at high- and low-risk zone net-pen sites throughout the Gulf of Maine. With the assistance of the National Marine Fisheries Services, which takes wild fish samples in the Gulf of Maine, and the U.S. Fish and Wildlife Service, which conducts a national wild fish health survey, routine sampling among wild and cultured salmon and nonsalmonid species is providing solid

evidence of the status of ISA and overall aquatic animal health status in the United States.

## Sample Specifications

Table 1 lists sample specifications for the ISA tests to be performed, the number of fish per pool, the required tissues, and the collection vessel used for each test.

## Identification of ISAv

To ensure that surveillance and diagnostic procedures are standardized throughout the industry, the following diagnostic methods to determine the presence of ISA are acceptable: histopathology, virology, RT–PCR, IFAT, and gross pathology.

All official diagnostic tests must be performed by a USDA–APHIS-approved laboratory. All ISA diagnostic test procedures will be performed according to the diagnostic test procedures approved by APHIS. Reasonable confirmatory evidence of ISA is based on clinical and postmortem findings in accordance with criteria described in section 2.1 of the ISA chapter in the OIE Diagnostic Manual for

**Table 1—Sample specifications for ISA Program-approved tests<sup>1</sup>**

Test	Tissue sample required	Collecting vessel/preservative medium
Histo-pathology	Kidney, spleen liver, gill pyloric cecae	Tube/10% formalin
RT–PCR <sup>2</sup>	0.25 cm <sup>3</sup> midkidney	Screw cap 2-mL tube/RNA
IFAT <sup>3</sup>	Midkidney impression	Frosted end slide
Virology in cell culture	Kidney, spleen gill, pyloric cecae	Specimen cup/phosphate-buffered saline

<sup>1</sup> All tests are for single fish in a pool.

<sup>2</sup> RT–PCR = reverse-transcriptase–polymerase chain reaction.

<sup>3</sup> IFAT = indirect fluorescent antibody test. All tests were performed according to the OIE Diagnostic Manual for Aquatic Animal Diseases.

Aquatic Animals, and the ISA virus is detected by performing the diagnostic tests mentioned above according to the OIE Diagnostic Manual for Aquatic Animal Diseases and other references established and filed with APHIS.

### **Cleaning and Disinfection**

Cleaning and disinfecting net-pen cages and associated structures are a vital part of the ISA control program. APHIS–VS had all 1,107 net-pen cages, primary nets, and predator nets cleaned by power washing, scraping, steaming, or all three methods (table 2). Nets were removed from sites and cleaned at an authorized facility. As provided in the interim rule, the agency paid the farmers for cleaning and disinfection costs where VS provided oversight of the process. Table 2 details nets cleaned and disinfected under APHIS supervision.

### **Eradication–Depopulation–Restocking Update of Salmon in Maine**

Currently there is no disease outbreak of ISA in Maine, and routine monthly surveillance samples have all tested negative. As of this writing, 17 sites holding 1,561,005 fish were depopulated. Of these 17, 8 were depopulated under the ISA Program and 9 were depopulated voluntarily before the December 13,

2001, declaration of emergency. All infected and exposed sites in the high-risk zone in the Gulf of Maine were fallowed for up to 90 days prior to restocking. Of those 17 sites, 6 have been restocked with a total of 1,800,320 smolts, and all of those smolts have been vaccinated against ISA.

A report of this current ISA update was submitted to OIE for redesignation of the United States as an ISA-free country on September 5, 2002. The United States is again officially ISA free.

### **Acknowledgments**

This report could not have been written had it not been for the efforts of many people from State, Provincial, Federal, and industry organizations, all working for a common cause related to the control of ISA. I want to acknowledge the individuals that I am aware of who contributed to the emergency declaration's becoming a reality and to the establishment of the ISA indemnity and control program:

USDA Secretary Ann Veneman  
 APHIS Administrator Bobby Acord  
 (former) VS Deputy Administrator Alfonso Torres  
 Commissioner George Lapointe, Maine DMR  
 Paul Waterstrat, Maine DMR  
 Senator Olympia Snowe  
 Congressman John Elias Baldacci  
 AVIC William Smith  
 ISA Program Manager Stephen Ellis  
 Economist John Green  
 Budget Analyst Toni Harris  
 Regulatory Writer/Editor Elizabeth Barrett

**Table 2—Cages and nets disinfected as of May 5, 2002 (N = 738)**

<i>Type and size</i>	<i>Cages</i>		<i>Nets</i>		<i>Total</i>
	<i>Number</i>	<i>Primary</i>	<i>Predator</i>		
12-m <sup>2</sup> steel	46	46	46	138	
15-m <sup>2</sup> steel	140	140	140	420	
20-m <sup>2</sup> steel	12	12	12	36	
24-m <sup>2</sup> steel	40	40	40	120	
70-m polar	65	65	65	195	
100-m polar circle	66	66	66	198	

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## Presentations From the Open Forum

**Note:** The first time each speaker addressed the forum, we are listing his or her affiliation in short form. For complete contact information, please see appendix 2 in the back matter.

### **Dr. David Scarfe**

American Veterinary Medical Association  
Richmond, VA

Good afternoon. I'm David Scarfe, and I will be moderating this last session. I hope that this will bring together many ideas and thoughts of many people with ISA experience. I think what we've experienced in ISA outbreaks over the last few years is very unfortunate, not only in one country, but a multitude of different places throughout the world. But countries have now responded strongly: aquaculture, which has always had a tendency to be the stepchild of agriculture, is now getting national recognition or equivalency.

This afternoon's session will be brief, to the point. We'll try to keep a lot of the session as focused as possible, but it may overflow into numerous topics. The intention for this afternoon is to try to focus on some innovative ideas about responding to ISA.

Obviously, some of this concept will apply to responses to other aquatic diseases. But I'd like you to focus on three elements of the response: prevention, control, and eradication.

In looking forward, utility should be a practical, theoretical, and problematic approach for us to consider in the future.

We've asked everybody wanting to offer comments for discussion to register a brief topic on cards, which have been grouped in an attempt to provide some structure and continuity. Initially registrants will be called on to make a brief presentation followed by comments from the floor before moving onto the next topic. If needed, an overhead projector will be available to illustrate points.

A court reporter will capture the salient points presented today to be added to the published proceedings after editing for clarity. I invite Jill Rolland to initiate the first topic for discussion.

### **Ms. Jill Rolland**

USDA-APHIS, Veterinary Services  
Riverdale, MD

On the west coast of the United States, where we have quite a bit of Atlantic salmon aquaculture in addition to five native species of Pacific salmon, many people are interested to know if our Pacific salmon stocks will be at risk should infectious salmon anemia make its way to the west coast. The U.S. Geological Survey's lab in Seattle is fortunate to have a BL-3 lab, a biosafety level 3 containment lab, which allows us to work with exotic pathogens. This enabled us to perform experiments testing the susceptibility of Pacific salmonids to the ISA virus. The species used in our experiments were chum, chinook, coho, and sockeye and for a positive control we used Atlantic salmon, which were flown in from the east coast. Two strains of ISAv were used, the North American strain CCBB and the Norwegian strain Bremnes.

The experiment was split into two trials. The first trial was carried out in the fall using three different viral doses of the Bremnes strain. During the experimental period, we sampled the mortalities for virus isolation and histopathology in addition to random samples taken from all species at specified time intervals.

The mortality rate in the Atlantic salmon was lower than expected, so we began a second trial in February. As only a limited number of fish remained, we limited our trial to using only one dose of virus, a high dose, and we used both the north American and Bremnes strains of virus. Again, we sampled both mortalities and took random samples throughout the experimental period.

In both trials, all Pacific salmon mortalities occurred within 2 days after experimental challenge. These fish had no signs of disease, as expected, and probably died from stress related to the challenge. The Atlantic salmon positive controls had low-level mortality in trial 1, but mortality reached 96 to 100 percent in trial 2. Therefore, it would seem that

Pacific salmonid species are relatively resistant to ISAv as compared to Atlantic salmon.

Whether or not these species can function as carriers and whether or not the virus is able to replicate in these species was not determined, nor was it the goal of this study. Instead we were attempting to answer the question of whether or not Pacific salmon are susceptible to the disease ISA, and based on our laboratory study, it would seem Pacific salmonids are resistant. We were able to get some low-level titers from some of the Pacific salmon at different time points, and whether we're reisolating a virus that we had already injected or we're actually seeing virus replication has not been determined.

**Dr. David Scarfe**

Thank you. If no one has comments related to that topic, we'll go on to the next topic. Ron Stagg would like to make some comments on management of ISA and brood stock farms.

**Dr. Ron Stagg**

Fisheries Research Services' Marine Laboratory  
Aberdeen, UK

The issue I want to talk about is how we might manage ISA if it occurs on a brood stock farm. The context of that discussion is that, in Scotland at least, we now have some different ways of managing brood stock. Some people grow the brood stock in cages and then transport eggs into fresh water, some transport fish into fresh water, and we also have a number of specialist brood stock farms in land-based systems. What would we do if we had ISA on those farms? The risk of spread of ISA is high if there is a risk of horizontal transfer to smolts. On the other hand, these farms are the future of the industry in the sense that they're developing and refining the brood stock and performance. So we have this paradox: these are very valuable fish, but if they contract ISA they are also a high risk for spreading the disease.

Have people thought about this in terms of management? The Royal Society of Edinburgh report on ISA raised this issue in Scotland, and I wonder what experiences are in other countries and how they might proceed to manage their situation.

**Dr. David Scarfe**

Are you suggesting something like specific pathogen free (SPF) certification, such as in some other agriculture industries, for example the swine industry?

**Dr. Ron Stagg**

Yes, possibly pathogen free. Maybe those farms need to have much higher security so that they prevent the possible ingress of ISA in the farms. But of course, that's a very expensive option.

**Dr. Paul Midtlyng**

VESO Veterinary Research  
Oslo, NO

You probably remember that there has been one case before of ISAv in Norway where the disease was present in a broodfish farm. At the time, the disease was not notifiable, so there were no instruments in place to prevent those eggs from being sold. What happened was that double disinfection procedures were applied before dispatching the fertilized eggs. This was in 1987, isn't that correct? I don't think that any epidemiologic evidence has since come up to suggest that there was a spread of infection from those positive fish.

Your lab, Ron, has recently published disinfection trials with the ISA virus; I believe it was last year [2001]. So in the present case, given the highly particular value of brood fish populations, I would let those live. I would question the justification of culling down superior—highly superior—brood stock for a disease like ISA. There are other means of control, which, to some degree, could remedy the problem.

**Dr. Ronald Roberts**

University of Idaho  
Twin Falls, ID

I'm from the University of Idaho, and I'm also director of Landcatch, Ltd., which is the largest independent land-based brood stock farm, I think, in the world. I certainly would like to comment on this because of concerns which we have, in Landcatch, having invested £5 or £6 million—that's \$10 million—into the development of highly selected brood stock only to find that the authorities insisted upon smolts being required to be slaughtered irrespective of genotype, from special lines, just for environmental surveys without any validity to distinguish between the elite ones, of which we are very much concerned, and the nonelite. And so I am very grateful that Ron Stagg highlighted this as a problem, and also that Ron is taking it very seriously.

The future of the industry—as with poultry and as with pigs—is going to depend upon the quality of brood stock and, specifically, pathogen-free brood stock. And if we are to be continually at risk from a condition which, in my view, is endemic (and I know Ron doesn't agree with that). . . . If this virus stays in our natural environment and if we could be under extreme sanction if at any time it happened to be found in our waters or in a single fish, then nobody is going to invest in the development of brood stock.

It has been argued that we should disinfect our common water. We have 6 tons/second of seawater going through our farm. I don't believe that a method for disinfecting those volumes of water exists, even in the U.S.

So I feel this is a very high-priority concern, and I would definitely agree with Dr. Midtlyng's statement that there is no evidence that this is an unmanageable disease. All our eggs are triple disinfected before they get to the customer. And the situation with regard to the importance of developing brood stock is such, and the nature of the ISA disease is such, that we cannot make the statement that we must put high-value brood stock at risk for a disease like ISA. That should be the message.

**Mr. Sebastian Belle**

Maine Aquaculture Association  
Hallowell, ME

My topic is somewhat related to this issue because the strategy that our growers have chosen to take with respect to brood stock is, no pun intended, not to put all of our eggs in one basket. They have decided to move brood stock to land-based holding farms and to locate those brood stock at more than one facility so that if a single facility is positive, at least you have the lines maintained in other facilities. However, that's a very expensive process, and we are far from achieving that at this stage of the game. It's a long-term goal on our part.

I do have some questions, though. A number of times during the symposium there were references to the detection of ISA in fresh water. There were a couple of different instances where it was shown, and I would like some clarification. If this relates to holding brood stock in land-based facilities, as well, what do people really know about ISA in fresh water? Are there, in fact, good, verified instances of positive clinical ISA in fresh water? Are we talking about just positive disease tests? And if that's the case, how does that relate to how we might manage the disease, as it were, in our brood stock programs themselves? How do you use that to your advantage?

**Dr. Ron Stagg**

In the fresh-water work we've done at the Fisheries Research Services' lab in Aberdeen, we've never observed clinical disease in fresh water, nor have we isolated ISAv. All of our positives have been determined by RT-PCR. I think we have to differentiate here between a background sporadic infection and the risk of a potential disease outbreak. The latter will depend on the form of the pathogen and the sustained nature of the infection.

In relation to brood stock farms, I would like to ask Mr. Belle whether they separate brood-stock holding areas from where they are growing smolts that come from the eggs from those brood stock.

I ask this in the context of the risk that might be presented in the event of an ISA outbreak and the possibility of cross-contamination in the event of these being shipped to on-growing farms.

**Mr. Sebastian Belle**

Well, we have different strategies in different facilities, and I think everybody accepts the fact that you have to try as much as you can to separate the brood stock from, obviously, the incubation of the smolt facilities. We have an added wrinkle in our case, and I think this is also the case in Scotland, to some extent: we also have farms that are maintaining brood stock in marine sites, and those sites, in particular, are obviously at great risk. We are very concerned about them. That's one of the reasons we went to the strategy of trying to divide the lines between different facilities.

But I absolutely support Ron Roberts' point, that the volume of water that is coming into the land-based facilities is enormous, and to ensure 100-percent disinfection of that incoming water is very, very difficult.

I mean, the real world often does not perform to the same performance standards that equipment manufacturers tell you they do. I think it's difficult to achieve that unless you build in multiple systems, and that causes you to have very high capital investments.

**Dr. Sandi McGeachy**

New Brunswick Department of Agriculture,  
Fisheries and Aquaculture  
Fredericton, NB

In New Brunswick, I think we have a number of land-based brood stock facilities now in place where the eggs and offspring haven't tasted saltwater. And what these facilities are doing is recirculating the water using low water flow, pathogen-free water. What they do with their siblings is send those out to saltwater to test their performance at sea, and they select their land-based siblings to obtain the better performance.

I know you don't get the same genetic gain as you would if you actually selected the actual fish performing in the saltwater, but within the brood-stock program in New Brunswick, that's how the plans are for training, in a biosecure source.

Again, this is spread across three facilities in Nova Scotia, one in Prince Edward Island, and a few in New Brunswick.

I know Roland Cusack is here. He might be able to speak about what is happening in the Nova Scotia industry. Those plans started a number of years ago, so we are now at the point of becoming self-contained or self-sufficient.

**Dr. Peter Merrill**

Micro Technologies, Inc.  
Richmond, ME

Keep in mind that there are no theoretical impediments to ISAv protection in fresh water because, in effect, any experiments in fresh water have demonstrated that fish can become infected and come down with clinical disease. I'm still not aware of any published reports, although I think [some investigators in] Norway reported some RT-PCR problems.

In Maine, with the majority of Maine companies following the ISA and the RT-PCR certification protocols, all brood stock are completely tested for ISAv at the time of spawning. The eggs are double disinfected, and before they are released from quarantine in a certified facility, the progeny from those eggs are retested for ISAv and then tested again before they're transferred to the mainland. So there's a fair amount of redundant testing involved. This doesn't complete the testing, but it does separate a particular type brood stock from blood-sampled brood stock.

**Dr. David Scarfe**

Thank you. If we can move to the next topic, Alasdair McVicar would like to comment on the current ISA situation in Ireland.

**Dr. Alasdair McVicar**

Canada Department of Fisheries and Oceans  
Ottawa, ON

I'm here to speak about ISAv in Ireland at the request of the Competent Authority there because they were unable to attend. They have isolated ISA virus on the SHK-1 cells and confirmed it by using IFAT and PCR. They used laboratories in Dublin (the Institute), the Marine Laboratory in Aberdeen, and the Community Reference Laboratory in Aarhus. A report was sent to the OIE Reference Laboratory in Oslo.

The Irish particularly wanted to acknowledge the cooperation obtained from these labs and especially Tore Håstein in Norway.

The date of suspicion was July 5, 2002, and the date of confirmation, August 1. All aquaculture facilities in Ireland have been tested for the presence of ISA using virus-isolation.

I'm just looking through their report to the Standing Veterinary Committee of the EC here to summarize the main points. The source of the virus is unknown, and the two affected rainbow trout farms, both in seawater, are isolated from other farms in Ireland. The authorities do not know when the disease was introduced. Strict biosecurity measures were in place in both sites on July 5.

At both sites, control zones were established according to the Directives. An effluent filtration system was installed in the processing units, with blood-water disinfection processing waste disposed of by rendering. The number of the fish in the two sites involved is 140,000 at an average weight of 2.5 kilos in one and at the second site, 220,000 at 400 grams and another 34,000 at 1.4 kg. One site has fish of mixed sizes. The other site, with market-sized fish, has actually been harvested.

Currently [September 2002], at the second site, they are doing a trial involving testing the pathogenicity of the isolated virus in Atlantic salmon. Until those tests are completed, the fate of the smaller fish on that second site is uncertain.

For this ISAv problem, there are continued higher levels of surveillance of the still-stocked affected salmon farm, and the nearest salmon farms. The way I see it, they are not finding any evidence of ISAv or disease in any of the farms.

**Dr. David Scarfe**

One question related to that: Is what you were describing specifically confined to salmon?

**Dr. Alasdair McVicar**

No. The tests were performed on trout, *Oncorhynchus mykiss*, only on trout. There were no salmon on these sites. These were two seawater rainbow trout farms.

**Dr. David Scarfe**

Did the animals show clinical signs, and do you have any idea what led someone to look for the ISA virus?

**Dr. Alasdair McVicar**

The fact that ISA was found in Scotland generated concern in Ireland and subsequent study at a high official level. That started the surveillance, and Irish authorities have since been doing this on a routine basis. So this culture, the presentation of virus, is what came up from that.

**Dr. David Scarfe**

Would anybody like to speculate with respect to Sebastian Belle's question concerning the presence of ISA in fresh water environments and any possible association between ISA in salmon and trout? If not, we will move on to a country's perspective of responding to the threat of an outbreak of ISA. Iain East has asked if he could make some comments about the Australian contingency plan for a potential ISA outbreak.

**Dr. Iain East**

Agriculture, Fisheries, Forestry—Australia  
Canberra, AU

I'd just like to make some brief comments. First off, in our case it's prevention and the steps we've taken to do that. Then second, I'll very briefly touch on some of the issues that were raised during the last day and a half.

In Australia, we've taken a proactive approach to try and deal with these things before they happen. It's interesting to hear that Canada is currently trying to develop an aquatic animal health plan. We have had one in place for the last 4 years. It's a plan that we have deliberately used to get industry's involvement because, as you all know, if you don't take industry along with you, plans are not worth the piece of paper they're written on.

Some of you may have picked up a copy of AQUAPLAN at the American Veterinary Medical Association's table earlier this week. If you didn't, copies are available on our Web site; or if you give me your business card, I can mail you a copy of the plan. It is designed to cover all aspects of health, as shown there, and the reason is that if you don't address all of those issues, then there will be gaps.

AQUAVETPLAN is our emergency management response plan. It's a series of manuals that cover Australia's predetermined response plan and how we respond to a range of diseases. To some extent it's still under development, but there are a number of components. Again, there were some CD-ROMs on the AVMA table earlier this week, and if you got one of those, congratulations; I think there were only 10. I can send those to you, or you can download the content from our Web site.

We have a plan under development for ISA, which includes a full scientific review of the disease, the principles of control and eradication, and the preferred control policy in Australia. When this is finished, it will be endorsed by all sectors of government and by the industry.

The value of this is that if we get the disease, we can pull the manual off the shelf, and we know what we're going to do. We don't want to spend days or weeks arguing with industry and governments as to what we're going to do.

Many of you will be aware of the import risk analysis for salmonid product and the dispute that we had with Canada. I don't want to take sides, but quarantine is one aspect of our policy, as is the surveillance program that we have up and running, and this predetermined response plan.

Switching subjects a bit, I would say that a couple of things I've heard today and yesterday are a bit worrying. They may be for your country a historical development, but I'd just like to give you a 30-second overview of how the management of our pearling industry works. The major component is what we call the "5 and 2 rule," which means that no two farms can be closer 5 km together unless there is a specific written agreement between the two farms that covers health management, and then we will allow them no closer than 2 km. Having salmon farms 300 m apart, as mentioned in a previous talk—how can I say it without starting an incident?—it's less than optimal.

In the Australian pearling industry, we also have predetermined zoning. Brood stock can come out of only one zone to a hatchery, and the offspring from that hatchery go back to that zone. There is no switching of animals across zones unless you are willing to put the animals at a quarantine site for 6 months and then have them health-tested after that.

There have been occasions where companies have taken the risk of doing that, and they've had their stock slaughtered out. This, we believe, is the only way that we can protect our industry, and these sorts of things do occur in our salmon industry. Some of you will be aware that we've had problems with aquabirnavirus in one region in Tasmania. That area is totally zoned off. You cannot take live animals out of there for any reason whatsoever. Product coming out of there has to be heads off, gills off,

guttled before it can move, and there is a very different level of surveillance in that area.

If we get disease, we insist on slaughtering out, and there is no government compensation at the moment in Australia. But this is the level, we believe, that we need to have to protect our industry from the sort of circumstances that other countries are unfortunately facing at the moment.

**Dr. David Scarfe**

Thank you. Dan MacPhee would like to comment on freedom from ISA.

**Dr. Dan MacPhee**

Maritime Veterinary Services  
St. George, NB

The question I want to raise is: Is Scotland free of ISA? The reason I ask is because, as I understand the program instituted in Scotland, if a farm was shown to be infected, the farm was required to remove all of their stock without compensation. Furthermore, farms within a 5-km zone were placed under increased surveillance. That would seem, to me, to create a huge incentive for farmers to avoid having ISA at all costs. First, because if they do report ISA, they're going to suffer a loss of millions of dollars which is almost certainly not insurable. Second, because they may bring the same fate upon their neighbors. Third, because in the coastal communities in which aquaculture exists, these actions may cause the loss of numerous jobs, and the effects would be felt throughout the community.

In one respect, I admire the strict regulatory approach that was adopted in Scotland because I support regulatory medicine where appropriate, and I believe it is appropriate in the case of ISA.

If governments are going to take such a drastic and Draconian action as eradication without compensation, I believe that they have a responsibility to determine whether or not the action they took was effective. The only means I know of to determine if such regulatory action was effective is to

measure the outcome. But in my experience, it may be very important to a farm to avoid the truth of the fact that they have ISA. Therefore, if there is a control program involving total eradication without compensation, the program probably won't have 100-percent industry support in terms of disease reporting. In that scenario, there needs to be a very, very intensive surveillance program before any conclusions can be drawn about the status of ISA in an area.

The first question I'd like to ask is addressed to the Scottish team: What are the details of the surveillance program instituted that has allowed them to declare that Scotland is free of ISA? Also, it has been mentioned that ISA is a reportable disease in Scotland. My second question is whether or not the disease is reportable by the farm or is it also reportable by others, such as private veterinarians or diagnostic laboratories? My third question is addressed to the audience in general: What is an adequate testing protocol in order to determine if an area is free of ISA, assuming that there will not be full cooperation on the part of the farms?

**Dr. David Scarfe**

Thank you. I see a tremendous number of issues being raised. Possibly most important: OIE has set some clear guidance for response to aquatic animal diseases; perhaps Tore Håstein would like to comment. I don't believe the OIE Aquatic Animal Code has suggested full guidelines for a valid declaration of ISA, pathogen or disease, freedom. As a general principle, continued active and passive surveillance are mandatory requirements for such a declaration. Concerning the Scottish suggestion of freedom (and I'll obviously defer to comments from the Scots), the information I read is that a statement of freedom originated from the Royal Society in Edinburgh, an independent scientific body who were requested to evaluate the information and provide an assessment of the ISA situation in Scotland.

There is undoubtedly a need for continuing intentional trade and recognition of disease-free

status, but there is going to have to be a definition of what constitutes freedom from an aquatic animal disease, particularly when there are very large reservoirs in wild aquatic populations, for which disease surveillance is very difficult.

**Dr. Tore Håstein**

National Veterinary Institute  
Oslo, NO

In reference to the OIE Code and Manual, OIE is currently writing a new schedule (introductory chapter) for testing in farming that will be published in the next edition of the Manual. The new general chapter for the Manual is currently being evaluated by an ad hoc group on risk analysis in aquatic animal health that will advise the OIE Fish Diseases Commission on amendments that need to be made to the surveillance and sampling methods in order to make the chapter as valid as possible. We sent it over to the risk analysis people to make sure it's okay.

**Dr. Ron Stagg**

I'll try to be very brief and factual. I think that's the best way to deal with this problem. First, I think we're talking about the eradication in Scotland of clinical disease, and I think we should take the message from the epidemiologic assessment we've made, the epidemic being the point source, and about the evidence of sporadic occurrence of virus in the environment. I think the two are very different. Second, the basis of our surveillance program is inspection for clinical disease. On that basis, the last clinical outbreak in Scotland was in February 1999. This approach reflects what Tore Håstein has just said about the methods available for screening for ISA.

In relation to the question whether industry would hide the presence of this disease, I think that's very unlikely given what happens in clinical cases of ISA and the way the mortality progresses. I think we also have to consider the very good working relationships with the industry and in particular the

joint response to dealing with the ISA crisis in Scotland. For example, in one case the farm was cleared while we were in the process of confirming. That showed industry's dedication to getting rid of this disease. So we not only have surveillance by the official service, we actually have surveillance by the industry *on* the industry, and I think we shouldn't forget that. And last, in response to the question of reporting, in Scotland it is a legal requirement for any person who suspects the presence of ISA to report this to the authorities.

**Dr. David Scarfe**

Thank you very much. Ron Roberts would like to address the issue of control programs without indemnification in the United Kingdom.

**Dr. Ronald Roberts**

I want to raise the question of control methods, or control systems, without indemnification for two reasons. I agree with Ron Stagg that if the lab is reporting ISA, it would be almost impossible to hide it, and certainly no one should do so. Also, virtually all fish farms in Scotland have their own veterinary surgeon and their own regular vet inspections. As a veterinary surgeon, I'm very aware of this. I have no doubts whatsoever concerning owners of ISA-affected farms not reporting to the appropriate authorities any real suspicion of any notifiable fish farm disease. On the other hand, there may be a need for acknowledging the fact that opportunities may need to be arranged for tracing back conditions which we don't know enough about yet, that could be confused with ISA, which could be in the background. I don't think anybody in the industry would jump to report such a condition to the department if there were no solid grounds for having made such a report. It's human nature to remain silent if one is not sure that he has good grounds for suspicion. Also, the results of reporting a disease are Draconian for the farm even if, in the end, the report proves to be negative.

I think that for the benefit of controlling this very, very serious disease on an international basis, there has to be the highest expectation of the earliest reporting. But in order for that to happen, there is a very, very strong case for removing some of the downside from the industry, particularly in relation to compensation, in order to stimulate the correct attitude of, “Don’t report it tomorrow; report it today. Don’t even think about it or sleep on it.” But there is also the much more significant aspect that it’s very easy to eradicate a disease by eradicating an industry. In Scotland, if things had gotten much worse, we would certainly have ended up with the complete eradication of an industry. As it was, many people lost their jobs and went into bankruptcy. Every company lost money somehow or other due to ISA. And I believe that in such situations, as with all the other notifiable disease considerations, depopulation has to be backed by a compensation policy. It really beggars belief that the country could spend £4 *billion* on the recent foot-and-mouth disease outbreak for two species which certainly, in Scotland, are lesser species than salmon aquaculture in terms of importance, while the government was not even prepared to spend probably £20 million on compensating people suffering through no fault of their own from ISA in the salmon industry—the largest single animal production industry in Scotland.

My second reason for supporting compensation is a moral one. It is a fact that, in the act of accession to the European Union, there are enshrined a series of human rights. One of these is that a person has the free right to use their possessions, unless the State requires to take them off them for some purpose that is above the level of the individual, i.e., is of national importance. In such an event, the owner of the taken property should be adequately compensated. The previous Lord Chancellor, the highest legal authority in the United Kingdom, has declared unequivocally that this is the case, and that it applies to the situation in the EU in relation to the ISA compensation issue. We have a situation where there is a capacity for compensation in the EU, and

yet no compensation is available in Scotland. Ron Stagg indicated that there is now a possibility of compensation. Without that, I believe investment is stymied. We, in fact, fail to maintain our investment at present, simply because of the total gamble that ISA represents.

I believe that compensation is something the whole international salmon industry has to address, and there should be uniformity of capacity for compensation. If it’s your fault if it happened or if you were wrong in early diagnosis, then you have to bear the brunt of the losses. That sounds fair enough, but totally innocent unfortunates should not suffer.

I do believe very strongly in both the need, for diagnostic purposes, of rapidity of reporting, and also in the need for justice in the form of compensation for rapid reporting leading to compulsory slaughter, as a result of the positive diagnosis of ISA.

#### **Dr. David Scarfe**

Thank you very much. Compensation or indemnification is a very interesting and important topic as it forms the basis for encouraging an adequate producer response but introduces the difficulty of determining who is financially responsible. In light of what we’ve heard earlier about what is occurring in Norway with insurers providing possible alternatives, the idea of having a producer “check-off” system (a mandatory set-aside from sales for such contingencies) in the United States may have to be considered fairly soon. Hugh Mitchell wanted to address indemnification.

#### **Dr. Hugh Mitchell**

Novartis Aqua Health  
Bothell, WA

I’m not sure if I can add any more than Dan MacPhee or Dr. Roberts did to the discussion. I’ve been associated with the industry over my entire career in North America, and I certainly have my fill of fish-disease regulations being applied without due compensation for the farmers. And I guess all I’d like to do is reiterate that I think it’s incredibly important,

both from an economic point of view, for the farmer but also for the accountability of the regulatory agency, to ensure that fair compensation indemnification is garnered before a disease regulation or program is put into place. I think it would be too cavalier to suggest that we can have a program in place that will work indefinitely, or work to eradicate diseases like ISA, without compensation. So I'm not going to carry on. I think that's been dealt with adequately by previous speakers.

**Dr. David Scarfe**

Thank you. Trevor Hastings would like to offer comments on diagnostic testing.

**Dr. Trevor Hastings**

Fisheries Research Services' Marine Laboratory  
Aberdeen, UK

We had some interesting papers during the workshop describing new cell lines for isolation of ISA virus, validation of methods, specificity, and so on. I'm also struck by the fact that there are no accepted methods for demonstrating the absence of ISA. In talking with colleagues at diagnostic laboratories in different countries, I'm struck by the differences that exist in criteria used for confirming the presence of ISA. For example, there are people who would not wish to confirm ISA unless disease was present. There are those who would confirm ISA on the basis of a virus isolation. And the consequences of these different huddle criteria for confirming disease can be extremely serious. I just think it would be useful for diagnosticians from different countries to communicate to each other and to try get organized on the standards they use for the diagnosis of ISA.

**Mr. Ragnar Thorarinsson**

ALPharma  
Bergen, NO

Well, there's talk about vaccinations already now in Europe. What I've been thinking about is basically a question to the diagnosticians in the audience: What

do you believe will be the value, or lack of value, with RT-PCR when our stocks of fish have been immunized with oil-based vaccines (containing inactivated whole virions as the ISA antigen) to all viral infections of the ISA virus?

**Dr. Peter Merrill**

Two comments. One, it's important to remember that the diagnosis of ISA disease among commercially raised fish is a right—in the United States, at least—exclusively reserved for veterinarians, who have the final say, whereas successful pathogen detection can be accomplished through any credible laboratory. I just wanted to mention that because I agree that there should be standards in place to harmonize methods by which a pathogen is detected. But there also has to be some leeway for the veterinarian to assess those findings in light of the clinical picture in which he needs to test the fish. As far as RT-PCR results showing up in postvaccinated fish, that actually hasn't been the case in my experience. I suppose the vaccine can affect it, but I did run a very large-scale field trial for an ISAv vaccine, and we tested a good number of fish at intervals of 1 day, 4 days, 7 days, 14 days, 28 days, and monthly thereafter, and did not find any evidence of positive results of RT-PCR in postvaccinated fish.

**Dr. Paul Midtlyng**

I would like to add that, should RT-PCR-positives occur in vaccinated fish, oil-adjuvant vaccine formulations, by themselves, have what you could call a marker which is the antibody response levels to those antigens present in the formulation. I haven't heard of similar antibody levels occurring in naturally infected fish. Neither would unvaccinated fish display any intraabdominal vaccine remains or adhesions, like we see in vaccinated individuals. So I think that for many practical reasons, fish that have received vaccine might be identified by a combination of tests against ISA virus and other constituents or antigens in those formulations. I would be surprised if any major misclassification would occur in such cases.

**Mr. John Reddington**

DiagXotics  
Wilton, CT

Yes, I think that there is an acute need for harmonization in these detection systems. And one of the issues is that there is fragmentation within the United States on what criteria this testing needs to meet, what approval processes need to be overcome. We've heard from several people that they get contradictory results from a plethora of labs.

My recommendation, at least as we move through the process, is that we don't focus so much on technologies as we start thinking about harmonization through a panel of characterized samples. I just hope to give those people who are interpreting the results some confidence of what abilities those labs have in terms of the reproducibility of the results that they're putting out.

**Dr. Øystein Evensen**

Norwegian College of Veterinary Medicine  
Oslo, NO

This might be a little bit outside of the area that we're discussing, but I want to comment on what was said about the importance of the hypervariable region of the HA protein, and possible implications of this in vaccine development. There's really a need to test if the variation seen in the various isolates correlates with the immune response and if it has any implication for protective immune response seen in the fish. It is not obvious that this is the fact.

**Dr. David Scarfe**

The virus we're dealing with is probably capable of rapid DNA change (evolution), typical of most influenza-type viruses, and I wonder to what degree we are discussing actual virus presence versus disease. And from a clinical point of view, I've always very clearly separated diagnosing disease (resulting in actual pathology) from virus identification. Even though these are highly interrelated and as diagnostic techniques for this disease become

refined, the epidemiologic understanding of this disease needs a clear distinction between presence of diseases and presence of pathogen. Trish Barbash wanted to comment on the disease in wild populations.

**Ms. Patricia Barbash**

U.S. Fish and Wildlife Service,  
Northeast Fisheries Center  
Lamar, PA

I would like to direct your attention back to wild fish for a few moments, and I want to share some information with you on some of the efforts my agency has made in evaluating the status of ISA in wild Atlantic salmon. In the Northeastern United States, my agency has been struggling to restore and/or maintain Atlantic salmon runs to about a dozen rivers. All but two of these rivers are in Maine. Salmon stocks have been designated under the Endangered Species Act as distinct population segments for eight rivers in Maine. All of those populations are perilously low, and our management of disease in the populations can be very tricky.

The best historical run remains the Penobscot River in Maine, which flows through Bangor. It can have a thousand salmon or more annually migrating from the sea. The fish are trapped at the Veazie Dam, and approximately half of the run is transported to a holding facility at the Craig Brook National Fish Hatchery and held for spawning in covered pools. Each pool will hold in excess of a hundred fish, depending on the run. The Craig Brook National Fish Hatchery also maintains endangered stocks of salmon from six rivers, held in separate rearing units, for the purposes of enhancement and restoration.

There is good deal of concern that sea-run fish carrying ISA from the ocean may put the endangered stocks at risk. Last year, we implemented a sampling regime on the Penobscot in order to accomplish several tasks: one, to determine the prevalence of ISA in fish caught from the Penobscot River during the upstream migration; two, to assess the risk of collecting these fish and holding them at a facility

which also rears endangered stocks; three, to assess the risk of transmission of the virus horizontally and vertically in fresh water; four, to track any detected infections as the fish remains in fresh water; and five, to determine best management strategies for minimizing the impacts of ISAv on these fish.

In 2001, we attempted to sample blood from as many incoming Penobscot sea runs as we could. We were only able to get 68 fish for reasons I'm not going to discuss here. The results were from RT-PCR, and some were other assays, but one blood sample in those produced a weak RT-PCR result, which was corroborated by another lab, and sequencing found it to be about 97 percent homologous to the Scottish/Norwegian strain of ISAv. The virus, however, was never isolated on cell-culture assays. After 1 month of isolation in a pool of fresh water, this fish was sampled again with negative results.

All the fish were sampled right before spawning; blood samples and all the results and cell cultures for RT-PCR were negative. In 2002, we attempted the same thing. We collected 126 samples from more than 380 fish brought to the facility. So far, all the tests that we've run are negative for ISAv. We do plan to test again before spawning.

It is impossible to draw conclusions from one RT-PCR finding from the 2001 sampling, although our results have raised some eyebrows and did, indeed, indicate that our wild salmon are most likely exposed to the virus at some unknown point while migrating and feeding in the ocean environment. Unfortunately, there's very scant information on the status of these fish when they're in the ocean.

Cooperation with the different resource agencies such as the National Marine Fisheries Service and the U.S. Fish and Wildlife Service and some of the State agencies is a good example of how we can continue to make efforts and learn about the status ISAv in wild populations.

**Dr. David Scarfe**

Thank you very much. I'd like to point out that this type of approach to active surveillance is absolutely

critical, albeit that it is fraught with all sorts of problems such as how to validly sample populations. Let's move on to looking at some vaccines; Kira Saloni has asked to make some comments.

**Ms. Kira Saloni**

Novartis Aqua Health  
Victoria, PE

First of all, I'd just like to invite you to the session [of the overall symposium] on vaccines, where I will present a paper on the efficacy of ISAv vaccines in fresh water. I'd like to break out a little bit of data from this presentation that are relevant to some of the things that we discussed here yesterday. It's mostly industry data about the experiences with depopulation and how that relates to vaccination status, so there are a few assumptions to be made. When you consider the presentation of the data I'm going to show you, the first assumption is that it was collected from farms and records of vaccinations, specifically with the assistance of the New Brunswick Salmon Growers Association. What makes this easier for us is because there are only two companies involved, so there are only two products, two different products being used on the fish at the fish-farm level. And we're basing this data on the assumption that all fish on exposed sites are all fish in one population, so we're dealing with results that are percentages calculated on numbers of fish, not sites. We are comparing the total number of fish depopulated to the total number of fish vaccinated. Vaccination status also has been assumed not to be biased to the criteria for depopulation. Most of the time, or at least in our experience, the veterinarians are not using vaccination status as an influence on the decision to depopulate.

In contradiction to the presentation yesterday, only 40 percent of the fish in the 2001 year-class are vaccinated, not 100 percent as was indicated. In the 2002 year-class, the majority has been vaccinated against ISAv. In 2001, approximately 60 percent of the population was not vaccinated specifically against ISAv.

Forte V1 is a Novartis Aqua Health product, and it represents only 23 percent of the population in the 2001 year-class. Our competitor, Bayer, is the other company in the Bay of Fundy marketing vaccines for fish, and we don't have any transparency with them, so I can't tell you what proportion of those fish are ISA-specific vaccinates. This [slide] is the ISAv status for the 2001 year-class from New Brunswick based on those data from industry. So the total number of fish in 2001 year-class on the positive sites, deemed positive by the criteria for ISAv status, is 22.7 percent. The total number of fish eradicated as of August 1, was 6.1 percent; "depopulated," I think, is a better term. The vaccination status versus depopulation status in the 2001 year-class for Forte V1, which is a vaccine specific for ISAv, Forte and Triple (multivalent bacterins), and a competitor vaccine are shown [in the slide]. As I said, we can't comment on the proportion in this population that are specifically vaccinated with ISAv. There is a fourfold decrease in the number of fish depopulated that were vaccinated with Forte V1 versus Forte-vaccinated fish.

We can state that vaccination reduces the risk of depopulation. This will be assessed further in my presentation [in the main symposium] tomorrow. And I would say that the situation for disease in the Bay of Fundy isn't particularly better this year than it has been in other years, but it's important to separate, when you speak of that, what the vaccination status of the population is. Thank you to Dr. Miller for the opportunity to present this in brief.

#### **Dr. David Scarfe**

Thank you very much. Trevor wanted to make some mention of field trials.

#### **Dr. Trevor Hastings**

It was interesting to hear that last report. It is in relation to that sort of thing that I want to speak. I'm struck by the absence of good field data on the efficacy of vaccines, and it seems to me that vaccine use is pretty unregulated. Virtually anybody seems to

be able to use vaccines or not use them according to their wish. Those who regulate the use of ISA vaccines don't appear to have specified that proper field trials be carried out, and I'd like to be corrected if I'm wrong. That's really what I'm talking about: whoever does allow these other ISA vaccines should place a requirement that they be properly evaluated rather than just used without advance testing.

#### **Dr. David Scarfe**

From the perspective of the American Veterinary Medical Association and the veterinary profession in the United States, we do have concern related to the understanding of efficacy of vaccines, specifically the degree of efficacy required of vaccines in the regulatory (licensing) process, and the public perception of how effective vaccines are. In my opinion, the false public perception of "once vaccinated, always free of pathogens or diseases" probably gleaned from the success against human polio and smallpox, is damaging to disease response. The AVMA is working with U.S. Federal agencies right now to try get appropriate labeling on vaccines and biologics to help correct these misperceptions.

#### **Dr. Paul Midtlyng**

I would like to comment on Trevor's statements. First, I'm not surprised about the quality of field data. I would rather say this problem is normal for field trials, unless the organizers put in a huge effort and manpower to follow up and manage their data to enable a high-quality efficiency evaluation. I think I've been perhaps one of the last persons conducting a huge field trial (on furunculosis) in a situation like this, but that was only made possible through full-time employment in followup. After 3 to 4 years of work, I was able to extract information out of which reasonably reliable results were obtained. But as far as I know, the ISA vaccinations have so far been operational, rather than for vaccine documentation purposes.

I think that for fish vaccines, their protective capacity must be demonstrated through controlled

experimental trials. The results may then be validated through field trials, which cannot, as I mentioned, be as well controlled as experimental work. I don't think that data coming out of the ISA field trials are worse than those coming out of most other field vaccine trials, but they are too uncontrolled to document the efficacy of the vaccines on their own.

**Mr. Pawan Agrawal**

Canadian Food Inspection Agency  
Ottawa, ON

I would like to clarify some of the vaccine-related issues. Our office is responsible for licensing veterinary biologics in Canada. In early 1998, we approved the use of autogenous ISA vaccines. We made certain exceptions to meet an urgent need for the vaccine at that time. But later, the ISA vaccines were licensed. They went through the laboratory approval process, which is similar to that for mammalian vaccines. There are no exceptions made.

Efficacy of the ISAv vaccine is based on the vaccination-challenge model in the lab situation. Right now, there's no requirement for a field efficacy for ISAv vaccine. I agree with the previous speakers in that it would be very difficult to generate the field efficacy data. There are several complications with that.

The first one is that the natural challenge is not very predictable. Another reason is, it's very expensive to run large-scale field efficacy studies. Also, the time required to generate the field efficacy data is very long. To generate valid data, we need to maintain vaccinated and unvaccinated fish in the same cage, and I don't think the fish farmers would like to do that.

During our licensing process, we consult with other agencies and departments, such as the Department of Fisheries and Oceans Canada, and also with the stakeholders as necessary. And our role is to approve the vaccine. It's other relevant Federal and Provincial authorities' responsibility to

decide whether to use the vaccine in certain situations or not. That is not our responsibility.

There were some issues raised about the potential for antigenic changes in ISAv. And early on, when we reviewed the ISAv vaccine submissions, we realized the need for addressing this issue. Therefore, we required that manufacturers reevaluate the efficacy of ISAv vaccine at an appropriate interval or whenever there's an indication that the vaccine is not protecting against the current ISAv strains. The manufacturers have to reevaluate the efficacy of the vaccine using the most recent field isolate.

For potency of current ISAv vaccine, each batch of the vaccine is tested by the manufacturer by a vaccination challenge method, and our office also evaluates it before we allow the distribution of ISAv vaccine for market. We welcome feedback from users.

**Dr. Peter Merrill**

I can give a little insight from the U.S. perspective, having some experience with innocuous ISAv vaccines. I want to point out that homogenous vaccines do not imply nor do they bear any requirement to [be effective in order to be] licensed vaccines under USDA regulations in the CFR. Field data may be offered in support of the efficacy, but it's not a requirement. And the only other comment I'd like to offer is that when field trials were begun, there was a very fine line set to both protect the proprietary interest of the vaccine manufacturer and still provide enough supportive information that can be shared and disseminated, to encourage the use of a particular vaccine. There are so many mechanical factors involved in running a field trial, particularly in the Passamaquoddy Bay–Cobscook Bay area, where there are differing approaches from government to government as far as protection and management of ISAv and ISA, respectively, that it's almost impossible to draw solid conclusions from field trials, even under the best of circumstances.

**Dr. David Starling**

USDA–APHIS, Veterinary Services,  
Center for Veterinary Biologics,  
Ames, IA

I'd like to address some of topics just brought up. One is the efficacy of vaccines. A lot of information is borne on the label. In most cases, licensed biologics here in the United States carry on the label the phrase, "as a significant aid in the prevention of." Veterinary Services' memorandum 800.200—it's posted on the Web site—can be read. By definition, if a particular vaccine is effective, under controlled conditions, there's a significant difference between a vaccinated group of animals and an unvaccinated group of animals when both groups are challenged by virulent organisms. The reason that wording is there is because the Virus–Serum–Toxin Act of 1913 specifically prohibits dangerous, contaminated, worthless products from being put into the market. The label endorsement saying a vaccine is "a significant aid in prevention of" a given disease is a clear indication to the consumer that this is not a worthless product. Products can be licensed and bear that label even though they've demonstrated a greater amount of efficacy. Most of this area is left to the manufacturer to choose, and they have reasons why they choose to go to a lower level of labeling. Also, the comment was made of the titers' not having efficacy testing and so forth. That is true. But according to section 113.113 of the Code of Federal Regulations, there shall be an expectation of efficacy.

**Dr. Hugh Mitchell**

I'd certainly like to dispel any notion that there's no willingness from the manufacturers, from veterinarians, or farmers to get field data. I mean, it always seems to be the Holy Grail. The question is: Does this product work in the real world? Well, one

of the problems in the real world is the inherent [variability] and it's one of the most fascinating and aggravating challenges to set up and run a field trial in the real world for the various vaccines. Many times, if you manage to accomplish that, the diseases change or the formulation of that particular vaccine changes. So I don't think it means we give up on doing field trials, but I do think it means we have to realize the limitations of such trials. Veterinarians, manufacturers, and farmers have to cooperate in unprecedented fashion to generate that kind of data where the willingness to get that data is there.

**Dr. Paul Midtlyng**

I'd like to start some discussion on the nonvaccination policy, or the prohibition of ISA vaccination. To put it bluntly, I believe there are no really good arguments to prohibit ISA vaccination as a part of ISA control programs. For the moment, we are talking about inactivated vaccines. We are having an uncontrollable source of potential infection in the wild. We all hope that this will trigger only occasional new outbreaks, but the risk is obviously there and cannot be completely controlled. The idea of keeping a sentinel population in such a situation, where the ISA diagnosis will first be confirmed after the animals have started to shed virus, is a terrible one for disease control. And this is unfortunately the current status of ISA carrier diagnosis—despite PCR and despite serology.

I would hypothesize that the current policy implemented by the EU is a kind of carryover from terrestrial animals, where farmed populations can be effectively isolated from the reservoir fauna. We really need to look into the details and the requirements necessary for nonvaccinated policy to be successful. I would very much appreciate discussing this controversial issue in greater depth, in order to optimize the ISA control strategies.

**Dr. David Scarfe**

I thank everyone for their thoughts and comment.



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**International Response to Infectious Salmon Anemia:  
Prevention, Control, and Eradication**

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