United States Department of Agriculture

Animal and Plant Health Inspection Service

United States Department of the Interior

U.S. Geological Survey

United States Department of Commerce

National Marine Fisheries Service

Technical Bulletin No. 1902

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

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.

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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 Epizootes 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^3-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 cytoxicity 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^3 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 ºC, 1 month at 4 ºC, or indefinitely at –20 ºC 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 ISAv 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 ISAv 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 ISAv 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 ISAv 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 ISAv 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 ISAv 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.

Acknowledgments

The author is indebted to Ms. Deborah Bouchard and Dr. Cem Giray of Micro Technologies, Inc., for their assistance with portions of this manuscript. Thanks also to Dr. Rocco Cipriano of the National Fish Health Research Laboratory, Kearneysville, WV; Dr. H. Michael Opitz of the University of Maine, Orono; and Dr. Beverly Schmitt of APHIS' National Veterinary Services Laboratories in Ames, IA, for editorial suggestions.

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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 Fredricton, 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 2×2 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 $(1 + 0.346*sigma^2)$, 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 2×2 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 pecent, 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)[1]	Population estimate (CI)[1]	Random effect: estimated ICC[1]	Random effect: *P* value	90% Predicted interval
Histology (positive)[2]	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)[3]	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[4])	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[4])	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[5])	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[5])	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[6])	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[6])	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

[1] CI = confidence interval; ICC = intraclass correlation coefficient.

[2] Suspects were considered positive.

[3] Suspects were considered negative.

[4] New Brunswick Department of Agriculture, Fisheries and Aquaculture.

[5] Research and Productivity Council.

[6] 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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