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

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