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# Comparison of lethal versus non-lethal sample sources for the detection of infectious salmon anemia virus (ISAV)

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ABSTRACT: The emergence of infectious salmon anemia virus (ISAV) in Canada and the USA has led to the establishment of ISAV surveillance programs for cultured Atlantic salmon (*Salmo salar* L.) and wild fish species, including Atlantic salmon. Current testing procedures for ISAV consist of viral culture, reverse-transcription polymerase chain reaction (RT-PCR) and indirect fluorescent antibody testing (IFAT), and require lethal sampling. As the focus of this study, blood was evaluated as a possible non-lethal sample source for ISAV diagnostic screening by viral culture and RT-PCR. Tissue samples (consisting of kidney/spleen for viral culture or kidney only for RT-PCR), blood and, to a lesser extent, mucus were tested from Atlantic salmon survivors of laboratory ISAV infection trials and moribund fish from marine salmon grow-out facilities participating in a USDA-sponsored surveillance program. The trial fish represented a potential carrier population, while the surveillance fish were composed of moribund individuals from ISA clinical sites. Sample sources and diagnostic techniques were compared. Blood compared well to tissue samples for viral culture and produced a greater number of positives than did kidney samples for ISAV detection by RT-PCR. RT-PCR using both kidney and blood samples was determined to be a more sensitive assay than viral isolation. Mucus did not perform well in either assay compared to the other sample sources. Blood appears to be a reliable non-lethal sample source for the detection of ISAV by viral culture and RT-PCR in both moribund and asymptomatic fish.

KEY WORDS: ISAV · Non-lethal sampling · Carrier populations · Blood · RT-PCR · Virus isolation



## INTRODUCTION

Infectious salmon anemia (ISA), induced by the viral causative agent infectious salmon anemia virus (ISAV), is a disease of variable infectivity and mortality affecting Atlantic salmon (*Salmo salar* L.). First observed in Atlantic salmon aquaculture operations in Norway in 1984 (Thorud & Djupvik 1988), ISA was later described from Scotland (Rodger et al. 1998, Rowley et al. 1999), New Brunswick, Canada (Mullins et al. 1998, Blake et al. 1999, Bouchard et al. 1999, Lovely et al. 1999), Nova Scotia, Canada (Ritchie et al. 2001), Cobscook Bay, Maine, USA (Bouchard et al. 2001), and from Coho salmon in Chile (Kibenge et al. 2001). The emergence of ISA in Atlantic salmon aquaculture operations located in northeastern Canada and the United States has led to the establishment of ISA management programs that have included monitoring of cultured Atlantic salmon stocks at active marine grow-out sites for the presence of ISAV. The diagnostic assays used for these programs have primarily focused on the testing of moribund fish and have utilized a combination of methods established for fish health testing (virus isolation by cell culture, molecular analysis through the reverse-transcription polymerase chain reaction [RT-PCR] and the indirect fluorescent antibody test [IFAT]), with kidney/spleen tissue the usual sample source specified (Fisheries

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and Oceans Canada 1984 revised 2004, AFS 2003, OIE 2003).

These established assays have also been employed for the detection of ISAV in potential carrier or asymptomatic populations of wild fish (including non-salmonids) and Atlantic salmon stocks used in stock enhancement programs. A procedure that does not require the sacrifice of the fish being tested, in particular wild Atlantic salmon or other valuable stocks, would be preferable to lethal sampling techniques. Therefore, a good, reliable, non-lethal sample source needs to be established for ISAV screening. Mucus has been indicated as a possible non-lethal sample source using experimentally exposed Atlantic salmon (Griffiths & Melville 2000).

This study examined the use of blood as well as mucus as potential non-lethal sample source alternatives to kidney/spleen tissues for the detection of ISAV by viral culture and RT-PCR in Atlantic salmon. IFAT was not included because of an apparent lower level of sensitivity in subclinically infected fish (Opitz et al. 2000, C. Giray et al. unpubl. data) and the unsuitability of blood smears for standard IFAT assays (Snow et al. 2003). Previous comparisons of methods used in the detection of ISAV from various organs of experimentally infected Atlantic salmon indicated that testing by RT-PCR was most effective in detecting ISAV-positive samples (Devold et al. 2000, Opitz et al. 2000, Snow et al. 2003). Furthermore, all samples that tested positive for ISAV by viral culture and IFAT were also found to be positive by RT-PCR (Opitz et al. 2000, Snow et al. 2003).

## MATERIALS AND METHODS

**Fish.** The samples, originating from multiple sampling events and laboratory submissions during 2001 and 2002, were presented to the laboratory from 2 distinct groups: 1-yr-old Atlantic salmon that were survivors of 4 ISAV co-habitation vaccine trials conducted in a saltwater wet-laboratory facility (experimentally exposed) and 2- to 4-yr-old salmon collected from ISAV-positive marine grow-out sites (naturally exposed). Although mortalities were also tested during the vaccine trials, the majority of the fish tested from the experimentally exposed group consisted of the survivors from control and vaccinated treatments sampled at the end of the challenge trials. Fish naturally exposed to ISAV were collected from Atlantic salmon marine grow-out sites experiencing mortality attributable to ISAV. Moribund fish demonstrating symptoms consistent with ISA were selected for testing. A portion of the samples was submitted to the laboratory blindly as part of a quality assurance (QA) testing program.

Tissues and blood were tested by viral culture and RT-PCR from a total of 308 Atlantic salmon, of which 147 fish were experimentally exposed, 159 naturally exposed, and 2 had no known exposure to ISAV and were used for negative controls. Prior to testing, all fish were evaluated as symptomatic (i.e. exhibiting gross pathologies consistent with ISA) or asymptomatic (i.e. no visible signs consistent with ISA). Although not pathognomonic for ISA, symptomatic gross pathologies included 1 or more of the following: pale gills, petechial hemorrhaging on the skin, hemorrhagic swim bladder, exophthalmia, darkly pigmented skin, ascites and anemia.

**Sample collection.** All samples from the various submissions and sampling events were collected in as consistent a manner as possible. Blood and kidney/spleen tissue samples were the primary sample sources selected for ISAV screening by viral culture. Individual blood and kidney samples were used for RT-PCR. Mucus was used for a portion of the testing, but was eliminated due to difficulties in processing, observed inhibitory effects on RT-PCR and the low level of correlation with results from other sample sources.

Tissue samples for virus isolation were collected by excising a 0.5 to 1 g piece of mid-kidney and spleen, and placing the tissues individually or in pools ranging from 2 to 5 fish in a pre-weighed container with 1 to 5 ml of phosphate buffered saline (PBS) at pH 7.2. Blood for viral culture was collected by caudal venipuncture, and 100 to 250 µl of whole blood from each fish was placed in 0.5 to 2.5 ml of PBS individually or as pooled (up to 5 fish) samples. Mucus for viral culture was collected from fish by scraping the surface of the skin using a sterile cotton tip swab and placing approximately 250 µl into 0.5 ml of PBS. Samples were stored at 4°C and processed within 24 h of collection.

Sample collection for RT-PCR was performed by excising a 100 to 200 mg piece of the mid-kidney and placing it into 1 ml of RNA*later*™. Blood and mucus samples were collected by placing 100 to 250 µl in 0.5 to 0.9 ml of RNA*later*™. Samples were kept at 4°C until processed (24 to 48 h), and stored at –20°C thereafter. All RT-PCR testing was performed on individual samples.

**Virus isolation by cell culture.** Tissue samples were weighed, homogenized and diluted 1:10 (w/v) in PBS. A second 1:10 (v/v) dilution of the tissue homogenates was then prepared in minimal essential medium w/Hank's balanced salts (MEM), supplemented with 2% fetal bovine serum (FBS), and each homogenate was filtered through a sterile 0.45 µm filter before inoculation onto cell lines. For blood and mucus, a 1:10 (v/v) dilution of the collected samples was prepared, and, as with the tissue homogenates, a further 1:10 (v/v) dilution in MEM and 0.45 µm filtration was performed.

This resulted in a final 1:100 (v/v) dilution of the original samples. Chinook Salmon Embryo-214 (CHSE-214) (Lannan et al. 1984) and Salmon Head Kidney-1 (SHK-1) cells (Dannevig et al. 1997) grown in 24-well plates were used for virus isolation. The cells were inoculated by removing the culture medium, placing 0.1 ml of the final 1:100 (v/v) dilution of the tissue, blood, or mucus sample into each of 2 wells of each cell line, incubating at 15°C for 30 min for virus adsorption, and adding 1 ml of the appropriate cell culture growth medium after adsorption (MEM w/5% FBS for CHSE-214 and Leibowitz' L-15 medium w/5% FBS for SHK-1 cells). Inoculated plates were incubated at 15°C and monitored for 28 d for any observable cytopathic effect (CPE). Isolation of ISAV was determined by the observation of CPE typical of ISAV in either or both of the cell lines and by its confirmation through RT-PCR.

**RT-PCR.** A 20 to 30 mg piece of kidney tissue was aseptically excised from the original sample, and up to 50 µl of blood or mucus in RNA*later*™ was extracted using the RNeasy Mini kit (Qiagen). Samples were homogenized in a sterile RNAse-free, 1.2 ml microfuge tube containing acid-washed 710 to 1180 µm (Sigma) and 3 mm (Kimble) glass beads using lysis buffer supplied with the kit. RNA yields were determined through UV spectrophotometry. RT-PCR amplification was performed using the GeneAmp EZ rTth RNA PCR kit per manufacturer's instructions (Applied Biosystems), with 25 pmol of each primer and 50 to 100 ng of RNA per 25 µl reaction. The ISAV 1D/2 primer set (Mjaaland et al. 1997, Blake et al. 1999), which amplifies a 493 base pair (bp)-band from Genome Segment 8, was used for all testing, while the FA3/RA3 primer set (Devold et al. 2000), which amplifies a 211 bp band, was used as a secondary tool. Amplification consisted of reverse transcription for 30 min at 60°C, 1 min incubation at 94°C, followed by 40 cycles of 94°C for 20 s and 59°C for 40 s and ending with a final extension for 7 min at 59°C. SYBR Green (Sigma) was used for staining of gels. DNA sequencing was performed on a select number of samples in order to confirm that observed bands corresponded to ISAV.

**Data analysis.** Statistical analysis was performed using S-Plus 6 software (Insightful). Data containing 2 categorical variables with 2 treatments each were analyzed using contingency tables of matched pair data and the McNemar's chi-squared test. Analysis involving 2 categorical variables with 2 outcomes was performed using Fisher's exact test.

## RESULTS

Among the group of 147 experimentally exposed Atlantic salmon, no significant difference was observed between blood and kidney/spleen as sample sources for the detection of ISAV by cell culture (Table 1). However, RT-PCR produced a significantly higher number of ISAV-positive results from both sample sources as compared to virus isolation ($p = <0.001$) (Table 1). At the same time, blood samples produced a significantly higher number of positive RT-PCR results than kidney samples ($p = <0.001$) (Table 1). Mucus samples were collected and tested from 54 experimentally exposed fish. These showed results that paralleled overall viral culture and RT-PCR results from kidney and blood samples (Table 1), but consistently produced a significantly lower number of positives than blood or tissue samples by both assays ($p = <0.001$); thus, tests with mucus were not pursued for the full duration of the study. The 2 individually tested negative control fish did not show CPE in cell culture and were confirmed negative for ISAV by RT-PCR.

While all salmon in the naturally exposed group were sampled individually for RT-PCR, some of the kidney/spleen and blood samples for virus isolation were collected through an ISAV surveillance program in pools ranging from 1 to 5 fish. Therefore, of the 159 naturally exposed salmon, 115 were tested by viral culture in a total of 24 pools, ranging from 2 to 5 fish in each, and 44 fish were tested individually. A significantly higher number of kidney/spleen samples compared to blood samples produced ISAV CPE during testing by cell culture ($p = 0.013$) (Table 1). Nearly all samples were ISAV positive by RT-PCR, regardless of whether blood or kidney was used as the source. RT-

Table 1. Overall virus isolation and RT-PCR results. Values represent number of individual samples or pools showing ISAV CPE or ISAV RT-PCR positive/total number tested (% ISAV CPE or RT-PCR positive). Individual or pooled samples were used for virus isolation, while RT-PCR was always performed on individual samples in the test. n/a: not tested by pooling; nt: not tested

| Sample | Experimentally exposed | | Naturally exposed | |
|---|---|---|---|---|
| | Virus isolation | RT-PCR | Virus isolation | RT-PCR |
| Kidney/spleen | 26/147 (17.7%) | 53/147 (36.0%) | 42/44 (95.5%) | 44/44 (100%) |
| Kidney/spleen (pooled for virus isolation) | n/a | n/a | 24/24 pools (100%) | 110/115 (95.7%) |
| Blood | 24/147 (16.3%) | 92/147 (62.6%) | 33/44 (75.0%) | 44/44 (100%) |
| Blood (pooled for virus isolation) | n/a | n/a | 20/24 pools (83.3%) | 112/115 (97.4%) |
| Mucus | 3/54 (5.6%) | 7/54 (13.0%) | nt | nt |

PCR showed a significantly higher number of positive results than viral culture when using blood as a sample source ($p = 0.0015$), but no significant difference was observed in detection capability of the 2 assays ($p = 0.134$) when using tissue samples (kidney/spleen for virus isolation and kidney for RT-PCR). Furthermore, in each case where ISAV CPE was observed in viral culture, a positive RT-PCR result was obtained from the corresponding sample, while the converse was not always true. Mucus samples were not tested from the naturally exposed group.

Atlantic salmon used for the study were also examined for gross pathologies consistent with ISA and characterized as symptomatic or asymptomatic. The group of 147 experimentally exposed Atlantic salmon consisted of 22 symptomatic and 125 asymptomatic fish. Both viral culture and RT-PCR produced a significantly higher number of ISAV-positive results in the symptomatic group than in the asymptomatic group, by both sample sources ($p = <0.001$) (Table 2). At the same time, ISAV was detected in a significantly higher number of fish by RT-PCR than by viral culture, in both groups, by both sample sources ($p = <0.001$) (Table 2). Mucus samples also produced a significantly higher number of positives in samples from the symptomatic group ($p = <0.001$), but overall detection was still significantly lower than in the other 2 sample sources ($p = <0.001$) (Table 2). The group of 159 naturally exposed Atlantic salmon consisted of 102 symptomatic and 57 asymptomatic fish. Since samples from symptomatic and asymptomatic fish were mixed within each pool, it was not possible to perform comparisons of viral isolation results among pooled fish. No significant difference was observed in RT-PCR results between the symptomatic and asymptomatic groups, but blood produced a significantly lower number of positive results by viral culture than by RT-PCR ($p = <0.001$) (Table 2). Overall, a significantly lower number of samples from the experimentally exposed asymptomatic salmon were positive for ISAV by the 2 diagnostic assays than in any of the other experimentally or naturally exposed groups ($p = <0.001$).

Table 2. Comparison of virus culture and RT-PCR results among ISAV symptomatic and asymptomatic salmon tested individually. Values represent number of samples showing ISAV CPE or ISAV RT-PCR positive/total number tested (% ISAV CPE or RT-PCR positive). Kidney/spleen samples were used for virus isolation; RT-PCR was performed on kidney samples only. nt: not tested

| Test/Sample | Experimentally exposed | | Naturally exposed | |
|---|---|---|---|---|
| | Sympto-matic | Asympto-matic | Sympto-matic | Asympto-matic |
| **Virus isolation** | | | | |
| Kidney/spleen | 19/22 (86.4%) | 7/125 (5.6%) | 40/42 (95.2%) | 2/2 (100%) |
| Blood | 18/22 (81.8%) | 6/125 (4.8%) | 32/42 (76.2%) | 1/2 (50%) |
| Mucus | 2/3 (66.7%) | 1/51 (2.0%) | nt | nt |
| **RT-PCR** | | | | |
| Kidney | 22/22 (100%) | 31/125 (24.8%) | 100/102 (98.0%) | 54/57 (94.7%) |
| Blood | 22/22 (100%) | 70/125 (56.0%) | 101/102 (99.0%) | 55/57 (96.5%) |
| Mucus | 1/3 (33.3%) | 6/51 (11.8%) | nt | nt |

## DISCUSSION

A higher proportion of naturally exposed Atlantic salmon than those experimentally exposed tested positive for ISAV by both viral culture and RT-PCR using both sample sources. It appears that testing of an ISAV clinical situation may result in a significantly higher number of corresponding positive findings through the 2 assays than testing of subclinical or carrier level fish. This may be explained by the composition of the experimentally exposed group of a higher proportion of asymptomatic fish (84%) than the naturally exposed group (5 to 36%). Also, naturally exposed Atlantic salmon originated from marine sites experiencing clinical ISA and therefore may have had higher viral loads, while the experimentally exposed fish were survivors of infectivity trials and thus potential carriers. Due to assay limitations, ISAV isolation by cell culture appears to be several orders of magnitude lower in sensitivity than the level of detection afforded with RT-PCR. All blood and kidney samples that showed ISAV CPE during this study tested positive for ISAV by RT-PCR, while the converse was not true. At the same time, virus detection through molecular techniques such as RT-PCR does not automatically or necessarily equate to the results produced by cell culture, which demonstrates the presence of viable, replicating virus. No conclusion may be drawn regarding the viability of virus detected by RT-PCR. Nevertheless, it is highly desirable that a combination of assays, rather than a single test, be employed for pathogen surveillance programs used for regulation or management strategies.

In this study, mucus did not produce ISAV-positive results at the levels that were obtained from other sample

sources. This may be a consequence of either the lowered persistence of virus in the mucus or the presence of inhibitory substances that interfere with testing protocols. It was also difficult to collect a sufficient volume of mucus without causing damage to the epidermal surface and leaving the site open to secondary infection. Still, further investigation of mucus as a potential non-lethal sample source for ISAV detection would be worthwhile.

Overall, this study indicates that blood is an equivalent and, with asymptomatic fish, a superior sample source to kidney when testing fish for the presence of ISAV by RT-PCR. Blood also generally performed well in ISAV isolation by cell culture, but with lowered sensitivity compared to kidney as a sample source when testing symptomatic naturally exposed Atlantic salmon. Since pathogen surveillance programs are designed for the early detection of ISAV, testing should preferably target its presence in asymptomatic fish before they present symptoms. When using blood as a sample source and RT-PCR as a test assay for ISA surveillance purposes, ISAV could potentially be detected well before disease outbreak, thus providing an advanced monitoring tool for the pathogen. Blood, as a non-lethal alternative, would also be a reliable sample source when testing wild Atlantic salmon stocks and in other situations where lethal sampling is not a desirable option.

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