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Infectious salmon anemia virus RNA in fish cell cultures and in tissue sections of Atlantic salmon experimentally infected with infectious salmon anemia virus

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Abstract. Current understanding of the etiopathogenesis of infectious salmon anemia (ISA) virus (ISAV) infection in fish comes mostly from virus detection in homogenized tissues taken from ISA-suspected mortalities. This study combined in situ hybridization (ISH) and histology to demonstrate viral RNA transcripts in different fish cell lines infected with ISAV and in tissues collected during the clinical phase of ISAV infection in Atlantic salmon. For this, a riboprobe to mRNA transcripts of ISAV RNA segment 8 was shown to detect viral mRNA in ISAV-infected TO, CHSE-214, and SHK-1 cell cultures. Specific hybridization was initially detected exclusively in the nuclei of infected cells, which is consistent with the nuclear transcription of orthomyxoviruses. For use of the riboprobe on fish tissues fixed in paraformaldehyde or formalin, the conditions used to permeabilize tissues before ISH (Proteinase K or Tween 20) were first optimized. Tissues were collected 15–20 days after challenge from 7 fresh mortalities of Atlantic salmon parr (~20 g) showing severe gross and microscopic lesions, consistent with ISAV infection. Reverse transcription–polymerase chain reaction on tissue pools confirmed the presence of ISAV in each of the 7 fish. Of the tissues examined in each fish, the heart and liver consistently showed the strongest hybridization signal and, therefore, the most in situ virus, which was located in the endothelium of small blood vessels and in macrophage-like cells.

Infectious salmon anemia (ISA) virus (ISAV), a member of the *Orthomyxoviridae* family,[21] genus *Isavirus*,[1] is currently one of the most important viral pathogens threatening Atlantic salmon (*Salmo salar*) aquaculture in the Northern Hemisphere. The signs of disease include exophthalmia, pale gills, ascites, severe hemorrhagic necrosis of internal organs, and high mortality.[11,25,33] Current understanding of the etiopathogenesis of ISAV infection in fish comes from virus detection studies using virus isolation in fish cell lines SHK-1[7] and CHSE-214,[4] reverse transcription–polymerase chain reaction (RT-PCR),[24] and indirect flourescent antibody test (IFAT).[13] Antigen studies[12] and electron microscopic studies have shown the virus to replicate and bud from endothelial cells of blood vessels and, in some cases, from the leucocytes of infected fish.[15]

The single-stranded RNA genome of ISAV comprises 8 segments of negative polarity, ranging in size from 1.0 to 2.4 kb with a total molecular size of approximately 14.3 kb.[6] The smallest segment, segment 8, was the first part of the ISAV genome to be cloned and sequenced.[24] Consequently, virus detection in clinical samples by RT-PCR is commonly based on detection of ISAV segment 8 sequences. This RNA segment in ISAV contains 2 overlapping open reading frames (ORF) estimated to encode proteins of 22 and 27.4 kD without splicing of transcripts.[29] Recently, the larger ORF of segment 8 was reported to encode the 24-kD major structural protein of ISAV, although the identity of this protein was not documented.[2]

Although the head kidney of ISAV-infected fish is generally considered as the organ of choice for virus isolation and the target organ that might harbor the most amount of virus,[9] different tissues from up to 5 fish suspected of ISA are usually pooled for the detection of the virus. Little effort has been made to substantiate the choice of tissues and whether virus presence in each individual tissue is correlated with lesions. The main goal of this study was to demonstrate the presence of viral RNA directly in fish tissues and cells during the clinical phase of ISAV infection in Atlantic salmon. To do this, it was necessary to develop an in situ hybridization (ISH) technique to detect gene expression of ISAV in different fish cell lines infected with this virus. This technique was then optimized for fish tissues and used to evaluate the distribution of virus in the affected tissues.

Methods

Cells and viruses. The ISAV isolate NBISA01[16] used for experimental infection of fish was grown and titrated in the TO cell line,[34] as described previously.[18] One hundred microliters of NBISA01 ISAV suspension with a titer of $10^{4.77}$ median tissue culture infec-

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tious dose ($TCID_{50}$) ml was used to inoculate the TO, CHSE-214,[22] and SHK-1[8] cell monolayers in slide flasks.[a] Some of the slide flasks were left uninoculated to serve as uninfected controls, whereas 1 flask of each cell line was inoculated with 100 μl of infectious pancreatic necrosis virus (IPNV), strain FVX73,[b] with a titer of $10^{7.5}$ $TCID_{50}$/ml, to check for specificity of the riboprobe. The presence of cytopathic effect (CPE) was monitored daily by microscopic examination. The monolayers of the different cell lines inoculated with ISAV were harvested at 1-day intervals. The IPNV-infected cell monolayers were also monitored for CPE and were harvested 3 days postinoculation (PI), when more than 60% of the monolayer had CPE.

Reverse transcription–polymerase chain reaction. Total RNA was extracted from 300 μl of cell lysate or tissue homogenate of the tissue pool with 700 μl of TRIzol Reagent,[c] according to the manufacturer's protocol. The PCR product used for preparation of the probe for ISH was obtained using the following primer pair[10] on ISAV RNA segment 8, 5′-GAA GAG TCA GGA TGC CAA GAC G-3′ (FA-3, nucleotide positions 342–363) and 5′-GAA GTC GAT GAA CTG CAG CGA-3′ (RA-3, nucleotide positions 532–552) (nucleotide positions based on GenBank Accession No. AF312317). One-step RT-PCR was carried out, as described previously,[18] using a commercial RT-PCR kit.[d]

Riboprobe synthesis. The RT-PCR product was purified from low-melting agarose gel using standard procedures[31] and subcloned in pCRII vector[e] using a commercial cloning kit, according to the manufacturer's protocols. The recombinant plasmids were transformed in *Eschericia coli*[f] competent cells, and transformants were screened by *Eco*RI digestion to observe the size of the insert DNA. To produce the DNA template for preparation of the riboprobe, the DNA insert was subcloned into a pGEM-3Z vector[g] having SP6 and T7 promoters using a commercial kit,[h] according to manufacturer's protocol. The recombinant plasmids in both forward and reverse orientation, with respect to the SP6 promoter, were isolated from *E. coli* using the standard alkaline lysis method.[3] The orientation of the DNA inserts in the plasmid was determined by restriction enzyme analysis. The antisense riboprobe, approximately 210 bases long, could be generated off the T7 promoter, as depicted in Fig. 1.

To generate the template needed for preparation of riboprobe, the plasmid DNA was digested with *Bam*HI for 2 hr at 37 C. The digested DNA was purified by phenol–chloroform extraction and precipitation with one-tenth the volume of 3 M sodium acetate, pH 5.2, and 2× volume of 100% absolute ethanol (i.e., ethanol precipitation in high salt). The in vitro transcription reaction was carried out in the presence of digoxigenin-11-deoxyuridine triphosphate (Digoxgenin-11-UTP[i]) using a commercial kit,[j] according to the manufacturer's protocol. In brief, the RNA synthesis reaction components, T7 polymerase and nucleoside triphosphates, were added to the linearized DNA with ribonuclease (RNase) inhibitor and incubated at 37 C for 2 hr. The RNase-free deoxyribonuclease was used to remove the DNA template. The digoxigenin-labeled negative sense RNA transcript was purified by ethanol precipitation in high salt and stored at −70 C until used. Digoxigenin incorporation, polarity, and specificity of the riboprobe, including a check for self-hybridization, were confirmed by hybridization against unlabeled homologous RNA transcript and total mRNA from ISAV-infected cell cultures in a Northern blot.

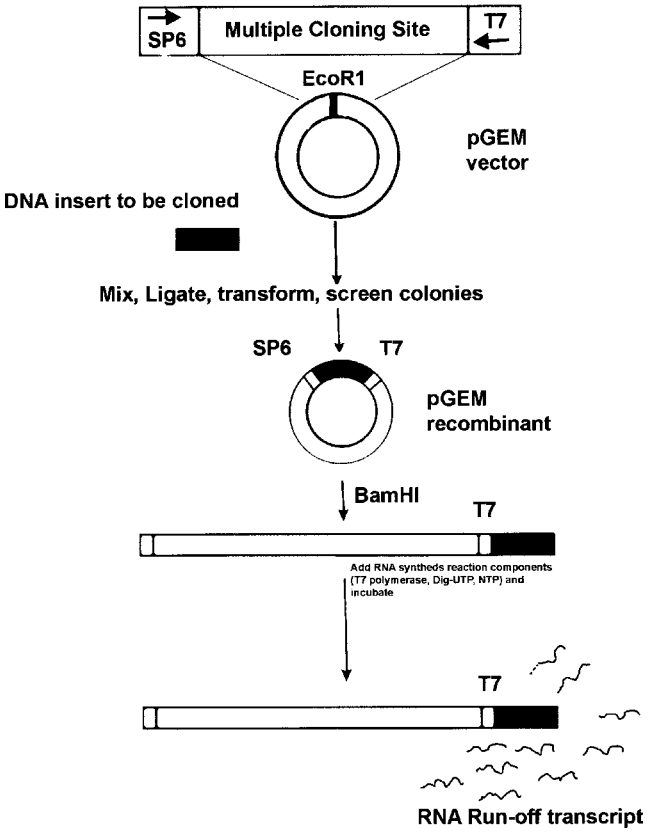

Figure 1. Diagram outlining the cloning of the ISAV RNA segment 8 cDNA in pGEM vector and subsequent in vitro transcription from the T7 promoter.

Experimental infection of fish with ISAV and tissue sampling. The fish used in this study were part of an initial population of 300 specific pathogen-free Atlantic salmon parr, St. John River stock, obtained from the Cardigan Fish Hatchery, Prince Edward Island, Canada. The mean weight and length of the salmon at the start of the experiment were approximately 20 g and 10 cm, respectively, in the freshwater phase. A sample of 6 fish from this population was screened for

ISAV by virus isolation attempts on the TO cell line and monitoring for CPE[18] and by RT-PCR to establish the ISAV-negative status of the stock before the virus challenge. The fish were maintained in the Aquatic Animal Facility of the Atlantic Veterinary College in 1-m-diameter, fibreglass-reinforced plastic tanks using a freshwater flow through system at a temperature of approximately 11 C. The experimental procedures used in this study were performed in accordance with the guidelines of the Canadian Council of Animal Care.[27]

For this study, the 50 challenge fish were removed from the stock-holding tank and anesthetized by immersion in an aerated solution of tricaine methane sulfonate (TMS-222) (100 mg/l). Each fish was then challenged by intraperitoneal injection of NBISA01 virus at a dose of $10^{4.8}$ $TCID_{50}$ in 0.2 ml of virus suspension and was then returned to the study tank. Sixty-five uninfected control fish were kept in another holding tank in a separate ''clean'' room. All tanks were checked twice daily for mortality; the fish were observed for abnormal behavior and external lesions. All dead fish were necropsied, and samples of heart, liver, spleen, gills, head and trunk kidneys, and pyloric caeca were collected aseptically in triplicate. The triplicate samples were treated as follows: 1 set of tissues was fixed in 10% formalin, a 2nd set was fixed in 4% paraformaldehyde, and a 3rd set of tissues was pooled in a sterile plastic bag and stored at −80 C for later use in viral analysis by RT-PCR. Two uninfected control fish were sacrificed every 2 wk during the ISAV experimental infection, and tissues were collected as for the fish mortalities. After 24 hr, the formalin- and paraformaldehyde-fixed tissues were processed in an automatic tissue processor[k] and were embedded in paraffin wax. Serial 5-μm sections of the fixed tissues were then stained with hematoxylin and eosin for light microscopy or were processed for ISH.

Ribonucleic acid ISH. The cell monolayers and fish tissues were prepared for ISH using standard procedures.[23] The slides of TO, CHSE-214, and SHK-1 cells were fixed for 30 min in freshly prepared 4% paraformaldehyde, dehydrated with increasing concentrations of ethanol (50%, 70%, 95%, and 100% ethanol), and stored at −70 C until needed. The slides of TO cells processed 4 days PI were used to optimize the ISH conditions. For this, each slide was divided into 6 wells. The prehybridization buffers and conditions were a slight modification of the method described previously.[19,20] The following concentrations of the riboprobe were applied to the different wells on the slides: 5, 2.5, 1.25, 0.625, and 0 ng/μl. Hybridization was carried out for 4, 8, or 16 hr at 37 C in a moist chamber. Posthybridization washes were as described previously.[19,24] The slides were washed twice at 37 C in 2× standard sodium citrate (SSC) (0.15 M NaCl and 0.015 M sodium citrate) containing 60% formamide and twice in 2× SSC followed by a single wash at room temperature in 2× SSC. The slides were then treated with 40 μg/ml RNase for 15 min at room temperature to remove unbound or nonspecifically bound riboprobe.

Five-micrometer sections of the paraffin-embedded, formalin- and paraformaldehyde-fixed tissues from ISAV-infected and control uninfected fish were placed on glass slides pretreated with 3-aminopropyltriethoxysaline[l] and deparaffinized by immersion in 2 changes of xylene for 5 min followed by rehydration in decreasing concentrations of ethanol for 5 min each and finally in diethyl pyrocarbonate–treated water for 5 min. The following permeabilization conditions of the tissues were examined to identify the one with the best ISH signal: incubation with 3% or 0.3% Tween 20%[c] in phosphate-buffered saline (PBS) (0.008 M sodium phosphate, 0.15 M NaCl, pH 7.2) or with 200 or 20 μg/ml of Proteinase K[m] in 50 mM Tris-Cl–5 mM ethylenediaminetetraacetic acid for 10, 20, or 30 min at room temperature or 37 C. Permeabilization was stopped by rinsing the slides with 2 mg/ml of glycine in PBS for 30 sec. Other prehybridization, hybridization, and posthybridization conditions were based on the optimized conditions established using the ISAV-infected TO cells.

Positive hybridization was detected in TO cells and tissue sections using a commercial digoxigenin label detection kit.[j] Slides were incubated for 1 hr at 37 C with alkaline phosphatase–conjugated anti-digoxigenin antibody diluted at 1:2,000, 1:1,000, 1:750, or 1:500 in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5). Signal was developed with the chromogen consisting of 0.375 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.188 mg/ml nitroblue tetrazolium (NBT) salt in detection buffer (0.1 M Tris–HCl, 0.1 M NaCl), pH 9.5, for 16 hr in the dark. Slides were counterstained with nuclear fast red[n] and mounted with Faramount aqueous mounting media[o] before microscopic examination. The slides of both cell monolayers and fish tissues were subjected to ISH using the optimized conditions. The hybridization signal was observed using a light microscope and was analyzed using Bioquant BQ-TCW 98[p] and Adobe Photoshop 5.5.[q]

Results

Development of ISAV-specific CPE. The ISAV-infected TO, SHK-1, and CHSE-214 monolayers were monitored daily for CPE before processing and storage. The appearance of CPE on the TO, SHK-1, and CHSE-214 cells was as described previously.[7,17,34] The timing of the development of the CPE in the different

Table 1. Development of infectious salmon anemia virus–induced CPEs in 3 fish cell lines.

Days postinfection	Intensity of CPE on the different cell lines		
	CHSE-214*	SHK-1	TO
1	−	−	−
2	−	−	−
3	−	−	+
4	−	+	++
5	+	++	+++
6	+	++	++++
7	+	++	+++++
8	++	++	ND
9	++	++	ND
10	++	++	ND
11	++	++	ND
12	+++	ND	ND
13	+++	ND	ND
14	+++	ND	ND
22	++++	ND	ND
28	+++++	ND	ND

* − = No CPE observed; +, ++, +++, ++++, +++++ = increasing intensity of CPE; ND = not done.

cell lines is summarized in Table 1. The ISAV-infected CHSE-214 cells did not show CPE until day 5 PI. The CPE then increased steadily until day 14 PI, when it covered more than 50% of the cell monolayer, as described previously.[17] The TO cells had visible onset of CPE at day 3 PI with complete loss of the monolayer by day 7 PI. The SHK-1 cells did not show a clear CPE pattern. No CPE was observed in any of the uninfected control cell monolayers of the 3 cell lines.

Preparation of an ISAV-specific riboprobe. The RT-PCR for ISAV RNA segment 8 yielded a 220-bp-long product. The product was cloned using the commercial cloning kit[e] and then subcloned into the pGEM-3Z vector.[f] The antisense riboprobe of 210 bases was produced by in vitro transcription of the linearized construct. The integrity of the riboprobe was checked by electrophoresis on a 2% RNA gel. The polarity of the riboprobe and the incorporation of the digoxigenin label were confirmed by hybridization against total mRNA from ISAV-infected cell cultures in a Northern blot (Fig. 2). Positive hybridization was detected only with total RNA extracted from ISAV-infected cells. Absence of hybridization by the riboprobe to the homologous unlabeled negative sense RNA transcript indicated that there was no self-hybridization with this riboprobe. The IPNV-infected and uninfected cell samples showed no signal (data not shown).

Optimization of ISH conditions for ISAV transcripts. The ISAV-infected TO cells used were fixed in 4% paraformaldehyde at 4 days PI when over 40% of the cell monolayer had CPE. The best hybridization signal (purple-blue color) was seen with a probe concentration of 5 ng/μl, hybridization time of 16 hours, and

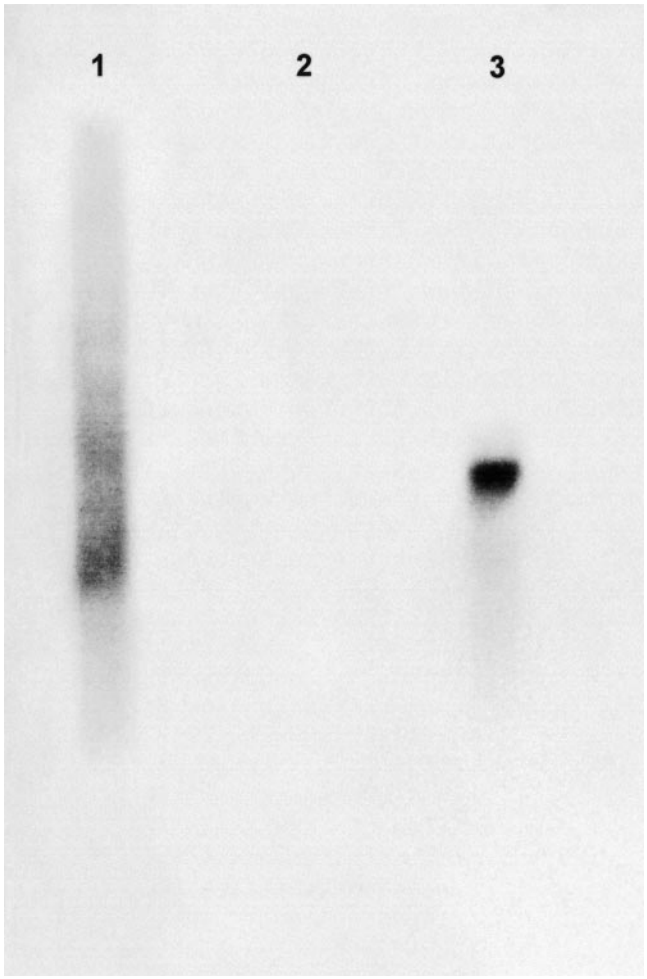

Figure 2. Northern blot hybridization using the ISAV RNA segment 8 riboprobe of negative sense. Lane 1, total mRNA from uninfected CHSE-214 cells; lane 2, unlabeled RNA transcribed from the 210-bp cDNA fragment of ISAV segment 8 and therefore of the same polarity as the riboprobe; and lane 3, total mRNA from ISAV-infected CHSE-214 cells, showing a strong positive hybridization band.

antibody dilution of 1:1,000 (Table 2). Because the ISH signal in tissues is influenced primarily by the permeability of the tissue[5] and conditions for permeabilization of fish tissues for ISAV studies have not been systematically investigated previously,[14] the fish tissues fixed in either formalin or paraformaldehyde in this study were initially optimized using 2 permeabilizing agents, a detergent (Tween 20) and a protease (Proteinase K), when used at different concentrations, durations, and temperatures. The results of the different permeabilization conditions for Proteinase K and Tween 20 are summarized in Tables 3 and 4, respectively. The best hybridization signal (++; strongly positive signal) in all the tissues fixed in either formalin or paraformaldehyde was seen when 20 μg/ml of Proteinase K was used at 37 C for 20 minutes and with an antibody dilution of 1:500. The time taken for

Table 2. Optimization of ISH conditions for infectious salmon anemia virus transcripts using a hybridization time of 16 hr.

Riboprobe concentration (ng)	Antibody dilution			
	1:500*	1:750	1:1,000	1:2,000
5.0	+++	+++	++++	+
2.5	+++	+++	+++	+
1.25	+++	++	++	+
0.625	+	+	+	−
0.0	−	−	−	−

* Intensity of hybridization signal: ++++ = very strongly positive; +++ = strongly positive; ++ = positive; + = weakly positive; − = no signal.

color development with the BCIP–NBT substrate was also examined, but no appreciable change was noted between the 6- and 16-hour intervals studied.

In situ hybridization in different fish cell lines. To demonstrate the specificity of the riboprobe for the detection of gene expression of ISAV, the riboprobe was applied to slides of the infected cell cultures. No hybridization signal was observed in the uninfected cell controls (Fig. 3a). Specific hybridization was initially detected exclusively in the nuclei of ISAV-infected cells (Fig. 3b, 3d). In both ISAV-infected TO and SHK-1 cells, the hybridization signal increased from day 1 PI, with progress of infection moving from nuclear to perinuclear cytoplasm at day 4 PI and becoming mostly cytoplasmic by day 7 PI (Table 5). In the ISAV-infected CHSE-214 cells, no hybridization signal was observed at day 1 PI with the riboprobe. From day 2, positive hybridization was seen in few cells, which increased as the infection progressed. By day 5 PI, the intensity of the hybridization signal was more, with more cells showing signal, and remained so (Fig. 3d) until day 14 when the last slide was sampled (Table 5).

Infectious pancreatic necrosis virus was used as a control that would cause CPE in the same cell lines that were used for ISAV and would therefore confirm the specificity of the riboprobe for ISAV mRNA in cell culture. Others have used tissues from fish infected with IPNV to demonstrate the specificity of ISAV riboprobes.[14] The appearance of IPNV-induced CPE on the CHSE-214 cells was as described previously.[35] More than 50% of the CHSE-214 and TO cell monolayers had IPNV-induced CPE by day 3 PI when the slides were processed for ISH. No hybridization signal was observed in IPNV-infected cell cultures using the riboprobe (data not shown).

In situ hybridization in fish tissues. Seven fish from those that died on days 15–20 after challenge were selected for histology and ISH on the basis of having gross lesions characteristic of ISAV infection.[33] The RT-PCR for ISAV was also positive for the 7 fish mortalities and negative for the 2 uninfected fish used as control. Sections of the heart, liver, spleen, head and trunk kidneys, gill, and pyloric caeca showed microscopic lesions, consistent with ISAV,[11,32,33] consisting mainly of hemorrhages, congestion, and multifocal coagulative necrosis of the liver (Fig. 4d). In addition, mild or low-frequency widespread endothelial hyperplasia was observed, which was most marked in the heart (Fig. 4a).

Using the ISAV riboprobe for ISH on the fish tissue, the hybridization signal was observed in all the tissues sampled. Signals were confined to the endothelial cells except in the heart, where they were present also in what appeared to be subendothelial macrophage-like cells (Fig. 4b), and in the spleen, where they were present in the macrophages or leucocytes. No hybridization signal was observed in areas of coagulative necrosis in the liver (Fig. 4d, 4e) or in viable hepatocytes (Fig. 4e). No hybridization signal was detected in any of the tissues from uninfected control fish (Fig. 4c, 4f).

Discussion

This study describes the production of a riboprobe specific for RNA segment 8 mRNA and its application to the detection by ISH of viral RNA in different fish cell lines and tissues from Atlantic salmon experimentally infected with this virus. The findings of this study show that mRNA transcripts of segment 8 were initially located in the nuclei of infected cells (Table 5), which is consistent with the nuclear transcription of orthomyxoviruses. An interesting observation in the ISAV-infected CHSE-214 cells was that the hybridization signal was initially focalized, similar to what was described previously for the CPE,[17] unlike those in ISAV-infected TO and SHK-1 cells, where the signal was dispersed throughout the cell monolayers. The detection of viral mRNA with the riboprobe by day 1 PI in the TO and SHK-1 cells and by day 2 PI in the CHSE-214 cell lines, before the onset of visible CPE, shows that this hybridization method is more sensitive for the detection of the virus than the virus isolation method alone. The lack of obvious CPE in the SHK-1 cells was attributed to loss of sensitivity of this cell line for ISAV at the higher cell passage levels used in this study.[30] Nevertheless, positive hybridization was

Table 3. Optimization of permeabilization conditions with Proteinase K for ISAV segment 8 riboprobe on tissues of fish experimentally infected with ISAV.

Fish no.	Serial section no.	Fixative*	Proteinase K (μg/ml)	Time (min)	Temperature (C)	Intensity of in situ hybridization signal in fish tissues†						
						Gill	Liver	AK	PK	Caeca	Spleen	Heart
AS-01	S4	PF	200	10	25	+‡	+	+	+	+	+	++
AS-03	S10	PF	200	10	25	+	++	+	+	++	+	++
AS-02	S8	F	200	10	25	++	++	+	++	++	++	++
AS-03	S2	F	200	10	25	++	++	ND§	++	+	++	++
AS-03	S1	PF	200	10	37	++	++	+	+	+	++	++
AS-06	S3	PF	200	10	37	−	−	−	−	−	−	−
AS-03	S3	F	200	10	37	+	−	−	+	−	+	+
AS-06	S6	F	200	10	37	−	+	−	−	+	+	+
AS-02	S9	F	20	20	37	++	++	ND	+	++	++	++
AS-01	S11	F	20	20	37	ND	++	+	ND	++	++	++
AS-08	S8	PF	20	20	37	++	++	++	ND	++	++	++
AS-07	S5	PF	20	20	37	ND	++	+	+	+	++	++
AS-07	S4	PF	20	30	37	ND	++	+	ND	++	++	++
AS-09	S5	PF	20	30	37	++	++	−	ND	++	+	++
AS-03	S9	F	20	30	37	++	++	ND	+	++	+	++
AS-06	S11	F	20	30	37	−	−	−	−	−	−	−
AS-06	S8	PF	20	30	25	+	+	−	−	+	+	++
AS-03	S5	PF	20	30	25	++	+	+	+	++	++	++

* PF = paraformaldehyde; F = formalin.

† Fish tissues are gill, liver, anterior kidney (AK), posterior kidney (PK), spleen, pyloric caeca, and heart.

‡ − = No hybridization signal obtained; + = weakly positive hybridization signal; ++ = strongly positive hybridization signal.

§ ND = No data available.

Table 4. Optimization of permeabilization conditions with Tween 20 for ISAV segment 8 riboprobe on tissue of fish experimentally infected with ISAV.

Fish no.	Serial section no.	Fixative*	Tween 20%	Time (min)	Temperature (C)	Intensity of in situ hybridization signal in fish tissue†						
						Gill	Liver	AK	PK	Caeca	Spleen	Heart
AS-02	S8	PF	3	10	25	+‡	++	+	+	+	+	++
AS-06	S9	PF	3	10	25	+	−	+	−	+	+	+
AS-03	S2	PF	3	10	37	+	+	+	−	+	++	++
AS-01	S3	F	3	10	37	−	−	−	−	−	−	−
AS-03	S4	F	3	10	37	++	+	+	+	++	++	++
AS-01	S10	F	3	10	25	ND§	+	+	+	+	++	++
AS-03	S6	F	3	10	25	++	++	ND	+	++	+	++
AS-03	S5	F	0.3	20	25	+	+	−	+	+	++	++
AS-06	S8	F	0.3	20	25	ND	+	+	+	−	+	+
AS-03	S4	PF	0.3	20	25	+	++	+	−	+	++	++
AS-09	S4	PF	0.3	20	37	+	+	+	ND	+	+	++
AS-03	S8	F	0.3	20	37	++	+	ND	−	+	−	++
AS-01	S9	F	0.3	30	37	ND	++	ND	+	+	++	−
AS-02	S10	F	0.3	30	37	−	+	−	−	+	+	+
AS-09	S8	PF	0.3	30	37	−	−	−	−	−	+	+
AS-08	S5	PF	0.3	30	37	+	+	+	−	+	++	++
AS-02	S3	PF	0.3	30	25	+	+	−	−	++	+	++
AS-01	S5	PF	0.3	30	25	ND	+	ND	+	ND	+	+

* PF = paraformaldehyde; F = formalin.

† Fish tissues are gill, liver, anterior kidney (AK), posterior kidney (PK), spleen, pyloric caeca, and heart.

‡ − = No hybridization signal obtained; + = weakly positive hybridization signal; ++ = strongly positive hybridization signal.

§ ND = No data available.

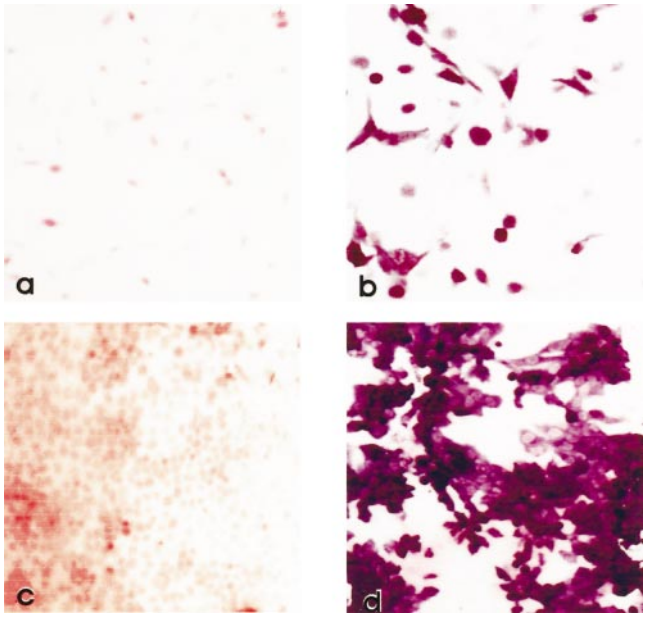

Figure 3. In situ hybridization using the ISAV RNA segment 8 riboprobe of negative sense in fish cells infected with ISAV. Specific hybridization resulted in a purple-blue reaction product in the nucleus and the cytoplasm of infected cells. **a**, uninfected TO cells showing no signal, **b**, ISAV-infected TO cells at day 1 PI showing a strong positive signal in the nuclei, **c**, ISAV-infected CHSE-214 cells hybridized without riboprobe showing no signal, and **d**, ISAV-infected CHSE-214 cells at day 10 PI showing strong positive signals in both the nuclei and the cytoplasm of infected cells.

detected in few of the ISAV-infected cells from day 1 PI. The ISH technique can be combined with virus isolation for earlier detection of the virus in clinical samples. The ISH technique will also show the distribution of the viral genetic material in infected cells. In this particular case, ISH was positive 2–3 days before the appearance of CPE in the cell lines studied.

Northern blot hybridization and ISH of IPNV-infected cell cultures were used to confirm the specificity of the riboprobe for ISAV RNA segment 8 sequences. The IPNV-infected cells showed clear CPE with loss of the monolayer in all the cell lines by day 3 PI when they were harvested, signifying that IPNV replicates in the fish cell lines used in this study. However, no hybridization signal was observed when the ISAV riboprobe was used for ISH on the slides with these samples.

Histologic evaluation, RT-PCR, IFAT, and virus isolation are commonly used to identify and diagnose the presence of ISA and ISAV in an infected tissue or fish. Electron microscopy has been used to show that the virus buds from endothelial cells in heart blood vessels and leucocytes.[15,26] The ISH technique will give a better spatial localization of the virus in the tissues/cells than electron microscopy. The optimal specific hybridization signal in tissue depends partly on the optimum permeabilization of the tissues/cells that harbor the viral genetic material.[5] In this study, the tissues were fixed in 2 fixatives (10% formalin and 4% paraformaldehyde) because a permeabilization condition that would give an optimum specific signal with either fixative was needed. Most field samples of suspected cases of ISA are fixed in formalin, hence the need to achieve conditions that might give the best hybridization signal for such samples. In this study, Tween 20 did not give a good hybridization signal when compared with Proteinase K (Tables 3, 4). In addition, positive signal was observed in at least 1 organ under all conditions when Proteinase K was used. The best signal demonstrating almost the same intensity in both paraformaldehyde- and formalin-fixed tissues was observed at 20 μg/ml Proteinase K at 37 C for 20 minutes. Paraformaldehyde-fixed tissues are assumed to give a better signal with ISH because paraformaldehyde does not cross-link proteins extensively so as to prevent the penetration of probes.[23] From this study, both paraformaldehyde- and formalin-fixed tissues gave reasonable signals under all conditions studied with either of the permeabilizing agents. Moreover, in this study both Tween 20 and Proteinase K effectively permeabilized the gill tissue, allowing it to be used in the ISH process. Others have reported difficulties with

Table 5. Distribution of the hybridization signal during the course of ISAV infection in different fish cell lines.*

Cell	1	2	3	4	5	6	7	8	9	10	11	12	13	14
TO														
Nucleus	++	++	++	++	+	+	−	ND	ND	ND	ND	ND	ND	ND
Cytoplasm	+	+	++	++	++	++	++	ND	ND	ND	ND	ND	ND	ND
SHK-1														
Nucleus	++	++	++	++	+	++	++	++	++	+	++	ND	ND	ND
Cytoplasm	+	++	++	++	++	++	+	++	++	++	++	ND	ND	ND
CHSE-214														
Nucleus	−	++	++	++	++	++	++	++	++	++	++	++	++	++
Cytoplasm	−	−	+	+	++	++	++	+	++	++	+	+	+	+

* ++ = Very strong positive signal; + = positive signal; − = no hybridization signal obtained. ND = not done.

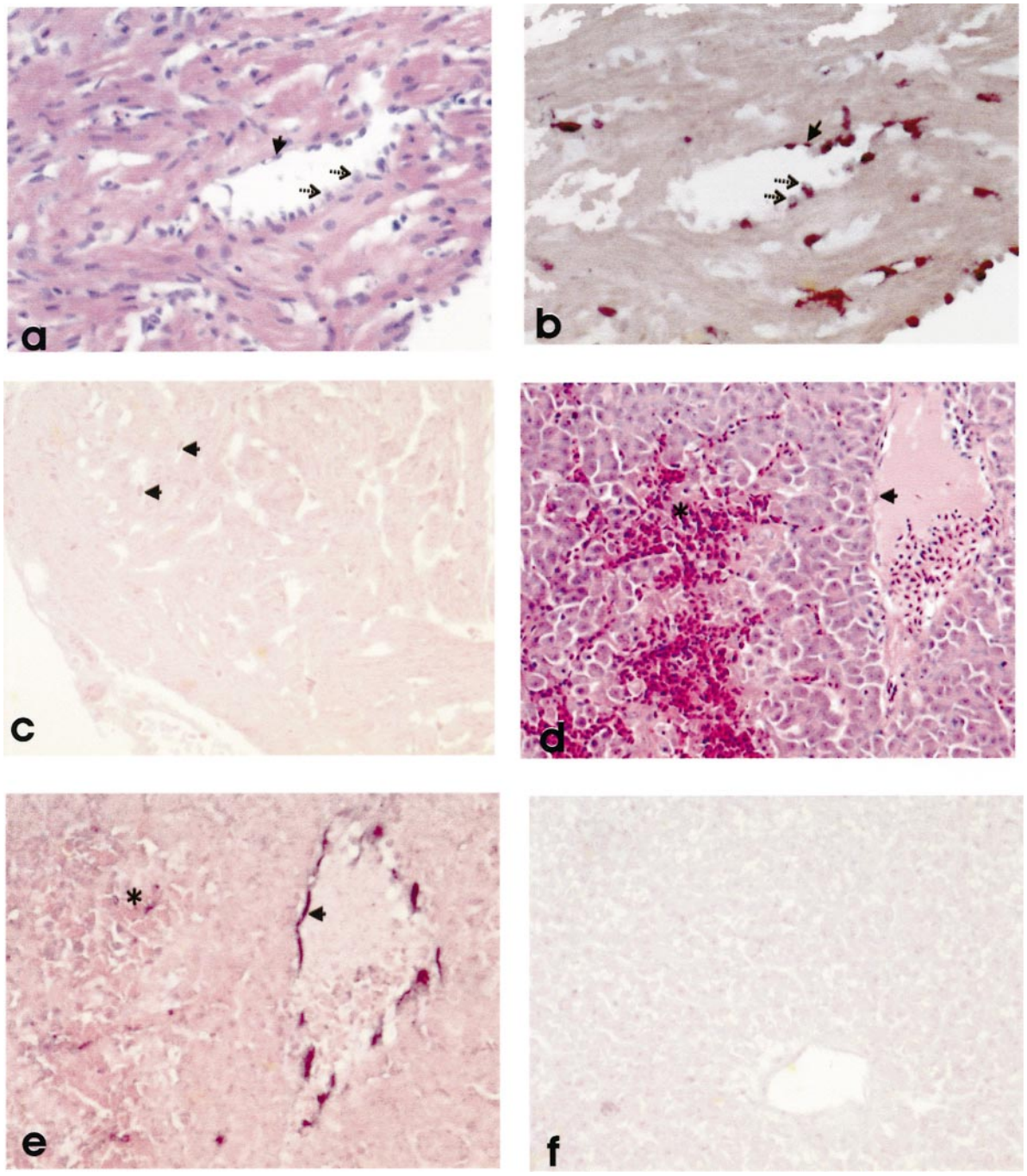

Figure 4. Hematoxylin and eosin (HE) and ISH with the ISAV RNA segment 8 riboprobe of negative sense for ISAV mRNA in tissues of Atlantic salmon mortalities after experimental infection with ISAV. No hybridization signal was observed in control tissue sections, in which the riboprobe was omitted during hybridization or in which the riboprobe was added, but the alkaline phosphatase–conjugated anti-digoxigenin antibody was omitted. Positive ISH signal resulted in a purple-blue reaction product in the cells. **a**, HE stain of the heart of infected fish showing widespread hyperplasia of endothelial cells (appearing flat, →) and possibly activated macrophage-like cells (appearing swollen, →). **b**, heart, numerous positive signals present in the endothelial cells (→) and macrophage-like cells lining the capillaries (→). **c**, heart of uninfected control fish, no signal observed in endothelial cells (→). **d**, HE stain of the liver of infected fish showing congestion and coagulative necrosis (*) and endothelial cells lining the blood vessel (→). **e**, liver, showing positive signal in the endothelial cells (arrow →) lining the blood vessel, but no signal in the area of necrosis (*). **f**, liver of uninfected control fish, no signal observed.

fish gill tissues, resorting to decalcification, which results in acidic hydrolysis of RNA.[14]

In all fish tissues used in this study, the cells with the ISH signal and therefore the target cells for the virus were endothelial cells and macrophage-like cells or mononuclear leucocytes. Surprisingly, the head and trunk kidneys, which are generally considered the target organs for ISAV,[9] did not show as much hybridization signal as did the heart and liver. Earlier studies[9] used infectivity of different tissues from infected fish inoculated into naive fish to show that the head kidney is the most important site early in infection for the replication of the virus followed by the liver, although these studies did not include the heart. Recently,[28] it was shown that use of nested RT-PCR on kidney tissues was the most sensitive method for the detection of the virus and that it correlated well with virus isolation and IFAT because most fish studied were positive by all 3 methods, but again the heart was not examined. From the ISH results of this study, the heart appears to be another organ to include for virus detection because most of its endothelial cells showed signals at optimum permeabilization conditions. Also under ''less ideal permeabilization conditions,'' positive signal was still picked up in the heart. This observation suggests the presence of more viral genetic material or replication of virus in the heart when compared with the head kidney. Alternatively, the heart may retain more of the viral genetic material in dead or moribund fish. These findings support other reports[12] that showed more pronounced reaction in the heart and kidney of infected fish by IFAT and electron microscopy.[15] With ISH, there might be some masking of the positive signal in the kidney by the brown pigments in melanomacrophages. The function of the melanomacrophages with respect to the internalization, processing, or replication of ISAV and the ability of these cells to maintain the same receptors or phagocytic properties as macrophages are as yet not known. The absence of a positive hybridization signal in any of the parenchyma cells is consistent with the targeting of the endothelial cells and blood cells for virus replication.

In conclusion, a riboprobe specific for mRNA transcripts of RNA segment 8 of ISAV has been produced, and the ISH technique has been applied to the detection of viral RNA in different fish cell lines infected with this virus. The findings in the infected fish cell lines are consistent with the nuclear transcription of orthomyxoviruses. The use of ISH with the riboprobe confirms that the endothelial cells are the main target cells for ISAV in infected fish, followed by the macrophage-like cells or mononuclear leucocytes. In addition, the heart was found to have high amounts of viral genetic material in dead fish and might be a valuable organ to include during screening or study of suspected fish by either virus isolation or RT-PCR. The endothelial damage in the heart and elsewhere may also play some role in the pathogenesis of the disease, as was suggested previously[32] for the liver. Further studies are in progress to correlate the microscopic lesions of ISA with the presence or localization of ISAV using ISH techniques.

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a. Nunc® Flaskette® Chamber slide, Nalge International, Naperzille, IL.
b. American Type Culture Collection, Manassas, VA.
c. Bio-Rad Laboratories, Hercules, CA.
d. Titan One Tube Kit, Roche Diagnostic Corp., Indianapolis, IN.
e. TOPO TA Cloning Kit, Invitrogen Life Technologies, Invitrogen Corporation, Carlsbad, CA.
f. *E. Coli* DH 5α, Invitrogen Life Technologies, Invitrogen Corporation, Carlsbad, CA.
g. pGEM-3Z Vector, Promega Corporation, Madison, WI.
h. Riboprobe Combination System SP6/T7 kit, Promega Corporation, Madison, WI.
i. Dig-11-UTP label, Roche Diagnostic GmnH, Roche Molecular Biochemicals, Penzburg, Germany.
j. Dig-UTP-Detection Kit, Roche Diagnostic Corp., Indianapolis, IN.
k. Sakura, Tissue Tek® VIP, Torrance, CA.
l. Sigma-Aldrich Canada Ltd., Mississauga, Ontario, Canada.
m. Invitrogen Life Technologies, Invitrogen Corporation, Carlsbad, CA.
n. Vector Laboratories Inc., Burlingame, CA.
o. DAKO, Carpinteria, CA.
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