

# Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection

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**ABSTRACT:** The emergence of infectious salmon anaemia virus (ISAV) in Canada and Scotland and frequent new outbreaks of the disease in Norway strongly suggest that there are natural reservoirs for the virus. The main host for the ISA virus is probably a fish occurring in the coastal area, most likely a salmonid fish. Since sea trout is an abundant species along the Norwegian coast, common in areas where ISA outbreaks occur, and possibly a life-long carrier of the ISA virus, a study was initiated to evaluate reverse transcriptase polymerase chain reaction (RT-PCR) for diagnosis of the virus in experimentally infected trout. Several tissues (kidney, spleen, heart and skin) were collected from the trout during a 135 d period. The following diagnostic methods for detection of the ISA virus were compared: cell culture (Atlantic Salmon Kidney, ASK cells), challenge of disease-free salmon with blood from the infected trout, and RT-PCR. The RT-PCR was the most sensitive of these methods. With the help of this technique it was possible to pick out positive individuals throughout the experimental period of 135 d. Challenge of disease-free salmon were more sensitive than cell culture (ASK cells). These 2 latter methods require use of the immunofluorescent antibody test (IFAT) or RT-PCR for verification of presence of ISA virus.

**KEY WORDS:** Infectious salmon anaemia virus · *Salmo trutta* · RT-PCR · Carrier

## INTRODUCTION

The emergence of infectious salmon anaemia virus (ISAV) in Canada (Mullins et al. 1998) and Scotland (Rodger et al. 1998) and frequent new outbreaks of the disease in Norway strongly suggest that there are natural reservoirs for the virus, probably in wild fish occurring in coastal area in the North Atlantic. The ISA virus has been shown to replicate in Atlantic salmon *Salmo salar*, sea trout *S. trutta* and rainbow trout *Oncorhynchus mykiss* and it cannot be excluded that the virus may also replicate in other species of *Oncorhynchus* (Thorud & Djupvik 1988, Hovland et al. 1994, Nylund et al. 1994a,b, 1995a,b,c, 1996, 1997, Nylund & Jakobsen 1995, Rolland & Nylund 1998). A few non-salmonid fish (turbot *Scophthalmus maximus*, sea bass *Dicentrarchus labrax*, goldsinny *Ctenolabrus rupestris*,

and ballan wrasse *Labrus bergylta* have been tested and found to be negative with respect to virus replication after challenge with ISA virus (Thorud & Torgersen 1994, Kvenseth 1998, authors' pers. obs.).

The classification of the virus has not yet been completed, but according to morphology (Hovland et al. 1994, Dannevig et al. 1995, Nylund et al. 1995a, Koren & Nylund 1997), biochemical characteristics (Falk et al. 1997, 1998), genomic organisation (Mjaaland et al. 1997), and the sequence of the RNA polymerase (Krossøy et al. 1999), it is most likely an orthomyxovirus. It has been shown that aquatic birds are the source of all influenza A viruses in other species (Fields et al. 1996). However, due to a low optimal temperature for the growth of ISA virus (Falk et al. 1997), the main host or reservoir species is probably a vertebrate with low body temperature and most likely a salmonid fish.

Atlantic salmon, sea trout and rainbow trout are all potential carriers or reservoir species. Outbreak of ISA

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in Norwegian salmon culture, in areas where rainbow trout is not present, indicates that this is not the main reservoir species along the Norwegian coast. With the exception of 1 individual (mature male several months after challenge) it has not been possible to show the presence of ISA virus in salmon that have survived an infection, not even after injection with stress hormones (prednisolone acetate) (authors' pers. obs.). This leaves the sea trout as the most likely carrier species in Norwegian waters. It is an abundant species along the Norwegian coast, common in areas where ISA outbreaks occur, and possibly a life-long carrier of the ISA virus (cf. Nylund et al. 1995b).

The aim of this study was to develop a rapid and sensitive RT-PCR for diagnosis of the ISA virus in carrier sea trout *Salmo trutta*. This method is compared with cell culture (Atlantic Salmon Kidney [ASK] cells) and challenge of disease-free salmon with blood from the infected trout.

## MATERIALS AND METHODS

Sea trout *Salmo trutta* and salmon smolts *S. salar* were supplied by 2 different smolt hatcheries close to Bergen with no history of disease. The sea trout stock were from river Sima (Hardangerfjorden), while the salmon stock were a commercial breed (originally from 2 different rivers on the west coast of Norway). Initial mean weight and length of trout and salmon were 42 g and 15 cm, and 48 g and 16 cm, respectively. The fish were acclimatised for 8 d to particle (20 µm) and UV-filtered (Katadyn J1/P, effect: 50 mW cm<sup>-2</sup>) seawater (34‰), kept at 12.5°C in 0.15 m<sup>3</sup> tanks (flow rate = 5 l min<sup>-1</sup>; 50 specimens in each tank), and fed commercial pellets twice a day.

The ISAV used in the challenge was collected in vacutainer blood-collecting tubes with sodium heparin in 1992 and 1993 from infected Atlantic salmon *Salmo salar* in 2 fish farms outside Bergen. The blood had been stored at -80°C before it was sonicated and sterile filtered (0.2 µm). The filtrate was diluted 1:1 in Hanks' balanced salt solution (HBSS). Each fish received an intraperitoneal (i.p.) injection of 0.2 ml of the virus solution and each control fish received an i.p. injection of 0.2 ml HBSS (Table 1). The fish were challenged on 16 April 1997. After 121 d, 14 August 1997, all groups were submitted to a latent carrier test (LCT), where each of the remaining fish received an i.p. injection of 0.2 ml prednisoloneacetate (10 mg prednisoloneacetate ml<sup>-1</sup>).

Several tissues (blood, kidney, spleen, heart and skin) were collected from the trout during the 135 d period (Table 1). The tissues were frozen in nitrogen and stored at -80°C for later use. Material from the following sampling dates were screened by cell culture, inoculation into disease-free salmon and RT-PCR: 2 May, 7 May, 9 June, 13 August and 29 August (Table 2). Tissues from the same fish were used in the screening by all 3 methods. However, the fish from each sampling date were pooled when screened by cell-culture and transmission into disease-free salmon, while the individual fish were kept separate in the RT-PCR screening.

The following measurements were made of the fish sampled: weight, length, and clinical signs of disease. Blood was collected for haematocrit measurements and blood smears made for study of blood cell morphology. A few selected fish (sea trout) were also examined bacteriologically by inoculation of kidney tissue on blood agar plates which were incubated for 6 d at 20°C, and on blood agar plates containing 2% NaCl which were incubated for 6 d at 15°C.

Table 1 *Salmo trutta* and *S. salar*. Experimental groups of sea trout and Atlantic salmon challenged with infectious salmon anaemia virus (ISAV) and control groups challenged with Hanks' balanced salt solution (HBSS). ST = sea trout challenged with ISAV, KT = sea trout challenged with HBSS, SS = Atlantic salmon challenged with ISAV, KS = Atlantic salmon challenged with HBSS. Number in the code is the tank number. Number of fish collected for screening at each date after the start of the experiment is shown. N = total number of specimens in each group

		Apr 28 Day 12	May 2 Day 16	May 7 Day 21	May 12 Day 26	May 20 Day 34	May 30 Day 44	Jun 3 Day 49	Jun 9 Day 54	Jul 8 Day 83	Aug 13 Day 119	Aug 29 Day 135	Dead	N
1ST	ISAV	5	5	5	5	5	5	-	5	-	5	9	1	50
2ST	ISAV	5	5	5	5	5	5	-	5	-	5	7	3	50
3ST	ISAV	5	5	5	5	5	5	-	5	-	5	5	5	50
6KT	HBSS	5	5	5	5	5	5	-	5	-	5	7	3	50
7KT	HBSS	5	5	5	5	5	5	-	5	-	5	5	5	50
8KT	HBSS	5	5	5	5	5	5	-	5	-	5	9	1	50
4SS	ISAV	-	-	-	-	-	-	-	-	-	2	3	45	50
5KS	HBSS	-	-	-	-	-	-	5	-	5	5	5	7	50 <sup>a</sup>

<sup>a</sup>The surviving salmon that were not collected during the experimental period (135 d) were taken out 2 mo later. No mortality occurred in this group after the termination of the other groups

Table 2. *Salmo trutta*. Material collected from the trout groups on Days 16, 21, 54, 119 and 135, after challenge was screened by cell culture, inoculation into disease-free salmon, and RT-PCR. The kidney (ST and KT) and blood (ST) tissues were homogenised, cleared by centrifugation, filtered (0.2 µm) and diluted 1:100 in PBS before incubation for 3 h on cell cultures with a mono-layer of ASK cells (80 cm<sup>2</sup>). The blood (ST) used in the challenge of disease-free salmon was diluted 1:7 with HBSS before intraperitoneal injection

Tissue	ASK cells (ml)	Inoculation into disease-free salmon (ml)	RT-PCR (mg)
Blood	10.0	0.2	-
Kidney	10.0	-	50-100

#### Establishment of a salmon kidney cell culture.

Pieces of head kidney from a sexually mature female Atlantic salmon *Salmo salar* L. were homogenised and centrifuged through Ficoll-Paque<sup>®</sup> (Pharmacia). The cells on top of the Ficoll fraction was collected and washed by centrifugation and resuspension, and finally resuspended in L-15 medium (BioWittaker) supplemented with FCS (10% v/v), gentamicin (50 µg ml<sup>-1</sup>) and L-glutamine (4 mM). The cells were cultured at 20°C in cell culture flasks (25 cm<sup>2</sup>, Nunc) and non-adherent cells removed after 24 h. The cell culture has now been passed 30 times and is in the text referred to as ASK cells. These cells show a distinct cytopathic effect (CPE), i.e. cell rounding and detachment from the substrate, 7 to 8 d after inoculation with the ISA virus.

**Virus inoculation.** Blood and head kidney tissues were kept separate, homogenised, and cleared by centrifugation before filtration (0.2 µm). The homogenates were diluted 1:100 in PBS and incubated for 3 h at 15°C in cell culture flasks with a mono-layer of ASK cells (Table 2). The inoculum was then removed and replaced by supplemented L-15 medium as described above, but with 5% FCS. The cells were incubated at 15°C for 12 d or until CPE could be observed. The medium from cells considered negative after 12 d was used as inoculum in a passage to new cells. The samples were passed 3 times before they were considered negative. A negative and a positive control cell culture were always included.

**Indirect immunofluorescence staining on cell culture.** The cells were washed 3 times in PBS pH 7.4 (0.1% Tween-20) and fixed in 80% acetone for 20 min. After complete drying the cells were blocked with 5% milk powder in PBS for 30 min. The primary mouse anti-ISA monoclonal antibodies (MAb) (3H6F8) diluted 1:150 was inoculated at 4°C overnight. The cells were washed 3 times with PBS (0.1% Tween-20) before the secondary antibodies fluorescein isothiocyanate (FITC)-labelled goat anti-mouse Ig diluted 1:100

was incubated at 20°C for 60 min. The cell nucleus was stained with propidium iodide (Sigma P-4170, 10 µg ml<sup>-1</sup>) for 1 min and washed with PBS Tween 3 times. The stained cell cultures were checked at ×1000 magnification in a Zeiss fluorescence microscope (Axioskop).

**Transmission trials.** To determine if the virus could be transmitted from the trout to disease-free Atlantic salmon (of the same origin as that used in the first challenge), blood samples collected from the trout groups (21, 54, 119 and 135 d after challenge) were used to challenge 4 groups of salmon, SS21, SS54, SS119 and SS135, respectively (Table 2). Blood from 5 trout in each group was mixed, sonicated, filtered, and diluted 1:7 with HBSS and 0.2 ml injected i.p. into 30 salmon in each of the 4 groups. Group SS21 was challenged on the 20 June 1997 while the other 3 groups (SS54, SS119 and SS135) were challenged on the 7 October 1997. The mortality in the 4 salmon groups was registered and samples for RT-PCR diagnosis of ISAV were taken from all 4 groups after 25 d.

**RNA extraction.** Kidney tissues from the salmon and the trout were homogenised in 1 ml of TRIZOL (Gibco BRL) reagent (50 to 100 mg of tissue / 1 ml TRIZOL). The homogenised samples were incubated at 20°C for 15 min (in some cases the samples were stored in TRIZOL at 4°C overnight). After incubation, 0.2 ml of chloroform was added per 1 ml of TRIZOL and the tubes were vigorously shaken (Vortex 15 s) and incubated for 5 min at 20°C. The samples were subsequently centrifuged at 12 000 × g for 15 min at 4°C. The upper aqueous phase (containing the RNA) was transferred to a fresh tube and the RNA was precipitated by mixing with 0.5 ml isopropyl alcohol. The samples were incubated at 20°C for 10 min and centrifuged at 12 000 × g for 15 min at 4°C. The RNA precipitated and formed a pellet on the side and bottom of the tube. The supernatant was removed and the pellet washed with 1 ml of 75% ethanol (the samples were centrifuged at 7500 × g for 5 min at 4°C). The RNA pellets were briefly dried (<10 min) and dissolved in 50 µl RNase-free water. Then the RNA solutions were immediately precipitated by adding 100 µl of 100% ethanol and 5 µl 3 M Na-Acetate (pH = 5.2). These solution were stored at -20°C for at least 1 h before centrifugation at 15 000 × g for 25 min. The RNA pellets were washed 2 times in 80% ethanol, dried for 10 min and dissolved in 50 µl RNase-free water. The double wash of the RNA was carried out to reduce the amount of possible inhibitors, from host tissues, to the RT-PCR. The quality and the quantity of the purified nucleic acids were determined by measuring the absorbance (A) at 260 nm/280 nm (A<sub>260</sub>/A<sub>280</sub>), in a spectrophotometer (Pharmacia Biotech Genequant II). These RNA-solutions were used for RT-PCR.

**RT-PCR.** About 1.5 µg total RNA + 1.5 µl (1.5 µg) random hexamers [pd(N)<sub>6</sub>] in a total volume of 10 µl were incubated at 70°C for 5 min and then cooled to 4°C. RT-mix containing 5.0 µl 5 × RT buffer + 1.2 µl 200 mM DL-dithiothreitol (DTT) + 2.5 µl 10 mM dNTP + 0.5 µl RNasin (20 U µl<sup>-1</sup>) + 4.3 µl dH<sub>2</sub>O + 1.5 µl (20 U µl<sup>-1</sup>) Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) were added at room temperature, making a total of 25 µl. This solution was incubated at 37°C for 60 min.

XPRIMER (Stew Scherer, Microbiology Department, University of Minnesota) software was used to identify primer pairs corresponding to Segment 8 (Accession no. AJ012285) of the ISA virus (FA-1/RA-1, FA-2/RA-2 and FA-3/RA-3) and to Segment 2 (Accession no. AJ002475), i.e. the RNA-dependent RNA polymerase (RdRp) gene (1.148A/1.148B) (Table 3). The DNA products resulting from amplification gave the expected size for all pairs of sense and antisense primers: FA-1/RA-1 = 416 bp, FA-2/RA-2 = 571 bp, FA-3/RA-3 = 211 bp, and 1.148A/1.148B = 383 bp. The most sensitive set of primers, FA-3/RA-3, corresponding to Segment 8, was used in the RT-PCR screening of the trout.

The PCR consisted of 2 µl of cDNA-solution that was added to 23 µl of reaction mixture consisting of 14.2 µl ddH<sub>2</sub>O, 2.5 µl (×10) running buffer, 2.0 µl (25 mM MgCl), 2 µl dNTP (10 mM), 1.0 µl of each primer (20 µM), 0.3 µl (5 U µl<sup>-1</sup>) Taq DNA polymerase (Pharmacia Biotech). The mixture was denatured at 94°C for 3 min and amplification was performed with 35 cycles of 94°C for 30 s, 59°C for 45 s, and 72°C for 90 s, followed by extension at 72°C for 10 min. The tubes were then held at 4°C. Amplification and reverse transcription were performed in a thermal cycler with a heated lid (TECHNE, Progene). The described protocol for RT-PCR was also compared with a commercial kit, a 1-step protocol for RT-PCR (Ready-to-go, RT-PCR

beads), from Pharmacia Biotech (cf. instructions included in the kit).

Aliquots (10 µl) of the PCR reaction mixture were electrophoresed in 1% agarose 1 × Tris-Borate-EDTA (TBE) gel containing ethidium bromide stain (Gibco-BRL) and photographed under UV transillumination. DNA molecular weight marker type 250 bp DNA ladder was applied to identify the size of the PCR products.

**Controls.** Primers for MHC-I (Accession no: L07605) were used as positive controls for the RT-PCR (Table 3). The DNA products resulting from amplification gave the expected product of about 464 bp (some individual variation), which is the length that will result from a RT-PCR of mRNA templates of MHC-I.

In addition to RNA from trout and Atlantic salmon that were not challenged with ISA virus, negative controls containing dH<sub>2</sub>O instead of RNA or cDNA were used to test each reaction mixture.

**Sensitivity.** An ISA positive cell culture supernatant was incubated at 4°C for 4 d before 1 ml of supernatant was taken out for end point titration in a 96-well culture plate, using an immunofluorescence assay (cf. Falk et al. 1998). The titration was done using ASK cells and the 50% tissue culture infective dose (TCID<sub>50</sub>) was estimated. The rest of the supernatant (49 ml) was precipitated with PEG8000 and centrifuged at 6000 × *g* for 1 h. The supernatant was then removed and the pellet dissolved in 1 ml Trizol for RNA isolation (procedure described earlier). The 49 ml supernatant gave a total of 4600 ng RNA or 184 ng µl<sup>-1</sup> after it had been dissolved in 25 µl RNase-free water. A 10-fold dilution series (37.0 ng to 3.7 fg) was made from the RNA and tested in the RT-PCR assay. The cDNA from these reactions were tested by the FA-3/RA-3 primers and 10 µl of each reaction was analysed by ethidium bromide-stained gel electrophoresis.

Table 3. Sequence, location, and characterisation of the primers tested for use in RT-PCR amplification of the eighth segment of the ISA virus and the RNA-dependent RNA polymerase (RdRp) gene. The sense (FA-1, FA-2, FA-3 and 1.148A) and antisense (RA-1, RA-2, RA-3 and 1.148B) primers were designed with the help of XPRIMER software. The first 6 primers correspond to Segment 8 (AJ012285) of the ISAV and the 4 others to the RdRp gene (Segment 2: AJ002475) and the MHC-I gene (L07605) of Atlantic salmon. The sequences are given in 5'-3' polarity

Primer	Sequence	Location on cDNA	% GC	Temp. (°C)
FA-1	5'-GAT CAG TCG AGC GAC GAT GAC-3'	198–218	57.14	66
FA-2	5'-GCG GAT TGT GTG TAG AGT TCT AG-3'	119–141	52.17	64
FA-3	5'-GAA GAG TCA GGA TGC CAA GAC G-3'	298–319	54.55	68
RA-1	5'-TTC TCG TCC AGC TCA TTC AGC-3'	593–613	57.14	66
RA-2	5'-CAA TTG GTC GAT TTC CCT GTT G-3'	668–689	45.45	64
RA-3	5'-GAA TTC GAT GAA CTG CAG TTA-3'	488–508	52.38	64
1.148A	5'-CAG GTC TAC TGT TGT AGT GAA GGC G-3'	1095–1119	52.00	76
1.148B	5'-CGA ACA TAG AGT TCA ACT CCA AGG TC-3'	1452–1477	46.15	76
MHC-IA	5'-CCG TCA GTG TCT CTG CTC CAG-3'	139–159	61.90	68
MHC-IB	5'-AAG TCA TCT GGG CAG CTC TCC-3'	582–602	57.14	66

In addition, various amounts of RNA (from 750 ng to 7.5 µg) from kidney tissues of ISA-challenged salmon were used as templates in a RT-PCR to get additional information about the sensitivity of RT-PCR detection of the virus. The RNA was isolated from an ISAV-infected salmon with a haematocrit of 33% and no clinical sign of disease. The cDNA from these reactions were tested by the FA-3/RA-3 primers and 10 µl of each reaction was analysed by ethidium bromide-stained gel electrophoresis.

The sensitivity of the PCR was tested by using various amounts of dsDNA from 2.2 pg to 2.2 µg as template for the FA-3/RA-3 primers. The dsDNA was produced by primer pair FA-2/RA-2, which give a sequence of 571 bp. The number of templates per µl reaction mixture was calculated according to the following formulae:  $\text{gram dsDNA} \times \text{Av/Mw}$ , where the gram dsDNA was measured at an optical density (OD) of 260 nm, and Av = Avogadro's number and Mw = the molecular weight of the 571 bp long dsDNA. Each nucleotide was given a weight of 325 Da. Each reaction was analysed by using 10 µl of the reaction mixture in ethidium bromide-stained gel electrophoresis.

**Specificity.** The primer sets (FA-1/RA-1, FA-2/RA-2, FA-3/RA-3) were targeted against the ISA virus sequence published by Mjaaland et al. (1997). The sequence is deposited in the EMBL, GenBank, and DDBJ database under Accession no. Y10404. However, due to errors in the sequence published by Mjaaland et al. (1997) (see also Blake et al. 1999), we re-sequenced the segment from their ISA virus isolate (Glesv er/400/90) and checked the primers against this new sequence (Accession no. AJ012285). The specificity of the primer sets was determined by sequencing the PCR products.

## RESULTS

The mortality was low in all groups after challenge, with the exception of the salmon group (4SS) challenged by the ISA virus. In this group 90% died within 32 d after challenge. There was no mortality in this group after Day 32. There were some mortalities (<10%) in the other groups after the start of the experiment. Bacteria were isolated from a few of the dead fish, but they were not among the known fish pathogens.

The haematocrit values dropped in all groups after the start of the experiment. However, the drop was largest in the groups challenged with the ISA virus. Haematocrit values as low as 1 (mean = 9) were measured in the salmon challenged by ISAV, while the lowest haematocrit in the control salmon was 30 (mean = 38, range: 30 to 47). After 21 d the mean haematocrit in the control trout (N = 15) was 39 (range: 29 to 46, variance: 28), while the haematocrit in the challenged

trout (N = 15) was 34 (range: 22 to 46, variance: 75). However, on Days 26, 34 and 44 after challenge the haematocrit values were about the same in the challenged and control trout groups. The mean haematocrits of control and challenged trout were significantly ( $p < 0.05$ , Mann-Witney *U*-test) different 119 and 135 d after the start of the experiment. In the control groups the mean haematocrit were 38 (range: 32 to 45, variance: 10) and 39 (range: 35 to 45, variance: 7) and in the challenged trout groups 34 (range: 28 to 42, variance: 17) and 36 (range: 28 to 45, variance: 23) 119 and 135 d after challenge, respectively.

## Cell cultures

The cell cultures inoculated with filtrate from blood and kidney tissue collected from ISA challenged trout 16, 21, 54 and 119 d after challenge were negative for ISA virus, i.e. no CPE was observed and the immunofluorescent antibody test (IFAT) staining was negative. The culture that was inoculated with kidney tissue collected from trout 135 d after challenge (16 d after injection of prednisoloneacetate) was clearly positive for ISA virus after the second passage, while the blood-inoculate from the same fish was negative. The positive control cell cultures were positive for ISA virus after 7 d (first passage). The positive cell cultures showed a distinct CPE and stained positive with the MAb against the ISA virus (IFAT). The negative controls were negative for ISA virus.

## Transmission from trout to salmon

The salmon (Groups SS21, SS54, SS119 and SS135) that were challenged with blood collected from the ISA-challenged sea trout 21, 54, 119 and 135 d after the start of the experiment became infected (determined by RT-PCR), but mortality only occurred in the salmon group challenged with blood collected after 21 d. All fish in group SS21 were dead 30 d after challenge showing clinical signs of ISA. They were also positive for ISA virus in the RT-PCR test. The Groups SS54, SS119 and SS135 were kept for 6 mo, but no mortality was observed. However, they were confirmed positive for the ISA virus 25 d after challenge with the help of RT-PCR.

## RT-PCR

### Sensitivity of detection

The cell culture titration (ASK cells) gave an infectivity titre of  $10^{6.4}$  TCID<sub>50</sub> ml<sup>-1</sup> supernatant. An amplifica-

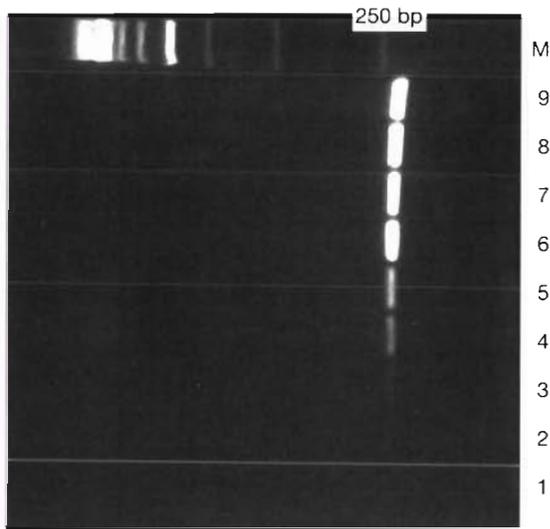


Fig. 1. Demonstration of the threshold of detection of ISA virus by RT-PCR using the FA-3/RA-3 primer set. Agarose gel showing RT-PCR amplification products (10  $\mu$ l) stained by ethidium bromide. DNA marker (M) is a ladder from 250 up to 3000 bp (the steps are 250 bp); Lanes 1–9 are RT-PCR products. The amount of RNA used in the RT-PCR ranged from 37.0 ng to 3.7 fg; (1) negative control, (2) 3.7 fg, (3) 37.0 fg, (4) 0.37 pg, (5) 3.7 pg, (6) 37.0 pg, (7) 0.37 ng, (8) 3.7 ng and (9) 37.0 ng

tion product could be visualised by ethidium bromide staining when as little as 37.0 fg of RNA were used template (Fig. 1).

When as little as 75 pg (1:10 000 dilution) RNA from ISA-infected salmon kidney tissue was used as template, the product could be visualised by ethidium bromide staining using 10  $\mu$ l RT-PCR product (Fig. 2). A similar result was achieved by using Ready-To-Go RT-PCR beads.

As little as 0.22 fg dsDNA in the PCR reaction using the FA-3 and RA-3 primers could be visualised by ethidium bromide staining using 10  $\mu$ l aliquots (Fig. 3). This would be about 300 templates of DNA in the 25  $\mu$ l reaction mixture. However, this band was very weak and in most cases the detection threshold would be between 300 and 3000 templates (cf. Fig. 3).

#### Specificity of the RT-PCR

In the specificity tests of the ISA virus RT-PCR assay, use of the sense and antisense primers on control trout and control salmon gave no amplification product. Sequencing of the PCR products from ISA-positive salmon and trout gave sequences that were similar to the target ISA virus sequence for all primer sets. The similarity of the 571 bp long sequence product of FA-2/RA2 primer set was 99.9% (1 nucleotide differ-

ent) compared to our sequence of Segment 8 of the Glesvær isolate (Accession no. AJ012285) of the ISA virus. The FA-1/RA-1 and FA-3/RA-3 primer sets anneal between the FA-2 and RA-2 primers and gave the same sequence similarity to the Glesvær isolate.

#### Diagnostic RT-PCR

Table 4 gives the results of the RT-PCR on kidney tissues from challenged and control trout. None of the control trout were found to be positive for the ISA virus. The percentage of positive trout 16, 21, 54, 119 and 135 d after challenge were 33, 45, 56, 64 and 67%, respectively. The signals detected by electrophoresis of the RT-PCR products from trout were significantly weaker compared to the signals produced by RT-PCR on RNA from ISA-infected salmon (Fig. 4).

#### DISCUSSION

PCR is becoming a more and more commonly used tool in detection and diagnosis of fish and shellfish pathogens (Arakawa et al. 1990, Nishizawa et al. 1994, Wongteerasupaya et al. 1997, Chi et al. 1998, Hiney & Smith 1998, Miller et al. 1998, Nilsen & Nylund 1998, Nunan et al. 1998, Oshima et al. 1998, Strømmen & Stone 1998), and universal RT-PCR protocols are being developed for human pathogens (Kuno 1998) and diseases of domesticated mammals (Nunez et al. 1998). The purpose of this work was to develop and test a specific and sensitive RT-PCR for detection of ISAV in experimentally infected carrier trout *Salmo trutta* and to compare this technique with isolation of the agent in host systems (ASK cells and Atlantic salmon). If successful, the detection of ISAV with the help of RT-PCR would have advantages over culturing the virus in host-systems in terms of a higher sensitivity and shorter detection time.

The available cell cultures (SHK-1 and ASK cells) that are used for isolation of ISA virus are also susceptible to IPN virus occurring in salmonids (Dannevig et al. 1995, authors' pers. obs.), which means that a CPE cannot be used as a safe diagnosis of the ISA virus (even if the CPE is different for ISA and IPN viruses). The cell cultures had to be stained with antibodies against the ISAV to verify presence of the causal agent. No CPE were observed in the cell cultures inoculated with homogenate from blood or kidney collected from trout 16, 21, 54 and 119 d after challenge with the ISA virus and the cell cultures stained negative (IFAT) for presence of ISA virus. The only trout groups that were positive for ISA virus were collected 135 d after ISA-challenge and 16 d after injection of prednisoloneac-

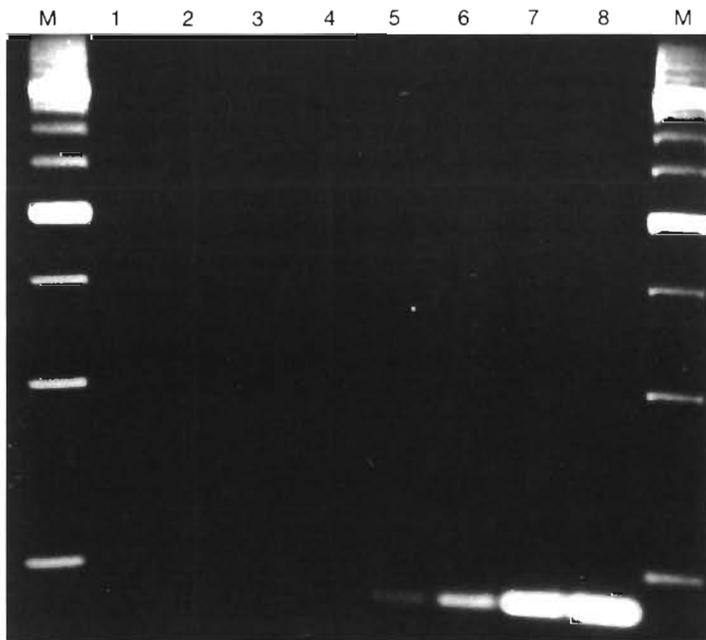


Fig. 2. Demonstration of the threshold of detection of ISA virus in kidney tissue by RT-PCR using the FA-3/RA-3 primer set. Agarose gel showing RT-PCR amplification products (10  $\mu$ l) stained by ethidium bromide. DNA marker (M) is a ladder from 250 up to 3000 bp (the steps are 250 bp); Lanes 1–8 are RT-PCR products. The amount of RNA used in the RT-PCR ranged from 750 ng to 7.5 pg; (1) and (2) negative control, (3) 7.5 pg, (4) 75.0 pg, (5) 0.75 ng, (6) 7.5 ng, (7) 75 ng and (8) 750 ng

etate. This shows that the ISA virus is present in the challenged trout throughout the experimental period, but the cell culture is not sensitive enough to be used for isolation and diagnosis of the virus in carrier trout. Most virus particles produced in a carrier trout are probably neutralised, and not infective. Injection of prednisoloneacetate probably influences the immune system in such a way that a higher number of infective particles are produced, i.e. enough particles to infect the cell cultures.

Injection of material which could contain ISA virus into Atlantic salmon may give the clinical signs most often observed in connection with an ISA infection, but a safe diagnosis would at least require the use of antibodies for a specific identification of the agent. The injection of filtered kidney homogenate collected from trout 21 d after challenge with ISA virus into disease-free salmon (Group SS21) resulted in 100% mortality. This shows that infective particles are present in the trout even if the number is not high

enough to infect the cell cultures. There was no mortality in Groups SS54, SS119 and SS135, but the salmon were positive (RT-PCR) for ISA virus 25 d after challenge. The difference between Group SS21 and the other 3 groups could be that they were challenged at different times due to practical problems with the availability of experimental tanks. The Groups SS54, SS119 and SS135 were challenged in October while SS21 were challenged in June. Previous studies have shown that a higher dose of ISA virus is necessary to cause mortality when the challenge is carried out in October to January (authors' pers. obs.). It is not known why the salmon seem to be able to better survive challenge with the ISA virus during the winter months.

The primer pairs appeared to be specific for ISA virus RNA, since RT-PCR products of expected size were amplified only when RNA isolated from ISA-infected trout or salmon were used as template. No amplification product was obtained when using RNA extracted from kidney tissues of healthy trout and salmon. The specificity of the primer sets was also confirmed by sequencing of the PCR products. The chosen primer sets are specific

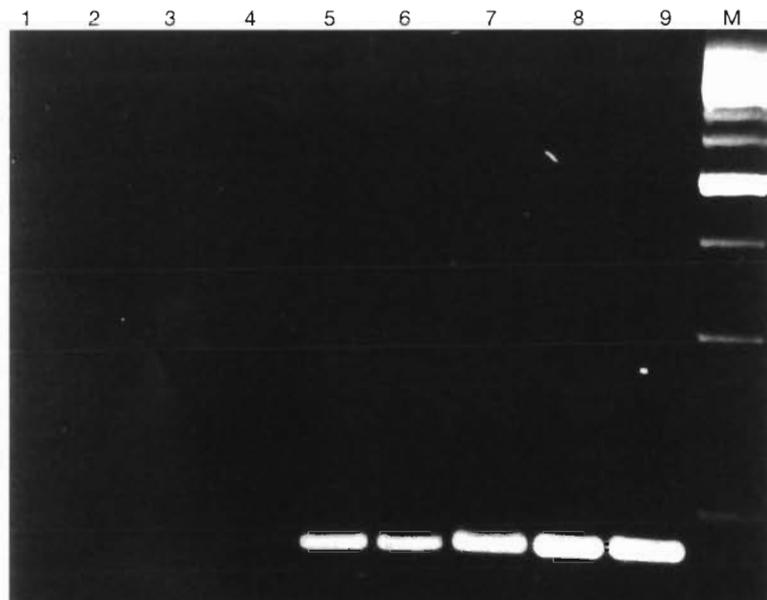


Fig. 3. Demonstration of the threshold of detection of dsDNA (made from ISA virus) by PCR using the FA-3/RA-3 primer set. Agarose gel showing PCR amplification products (10  $\mu$ l) stained by ethidium bromide. DNA marker (M) is a ladder from 250 up to 3000 bp (the steps are 250 bp). Lanes 1–9 are PCR products. The amount of dsDNA used in the PCR ranged from 2.2 pg to 0.0022 fg; (1) and (2) negative control, (3) 2.2 ag, (4) 22.0 ag, (5) 0.22 fg, (6) 2.2 fg, (7) 22.0 fg, (8) 0.22 pg and (9) 2.2 pg

Table 4. *Salmo trutta*. Results of the RT-PCR screening of the control and ISAV-challenged trout using the FA-3/RA-3 primer set. The fraction of ISAV-positive fish in the RT-PCR screening is shown. ST = trout groups challenged by ISA virus. KT = groups of control trout. Number in the code is the tank number

	May 2 Day 16	May 7 Day 21	Jun 9 Day 54	Aug 13 Day 119	Aug 29 Day 135
1ST	1/4	1/3	2/3	3/4	3/4
2ST	1/2	1/3	1/3	2/4	1/2
3ST	1/3	3/5	2/3	2/3	2/3
6KT	0/3	0/3	0/3	0/3	0/3
7KT	0/3	0/3	0/3	0/3	0/3
8KT	0/3	0/3	0/3	0/3	0/3

for the ISA virus and will not pick up any sequences from the fish genome. However, it is not known if the primers can be used for differential diagnosis of viruses closely related to the ISA virus, but they will not pick up sequences from the IPN virus, which is the

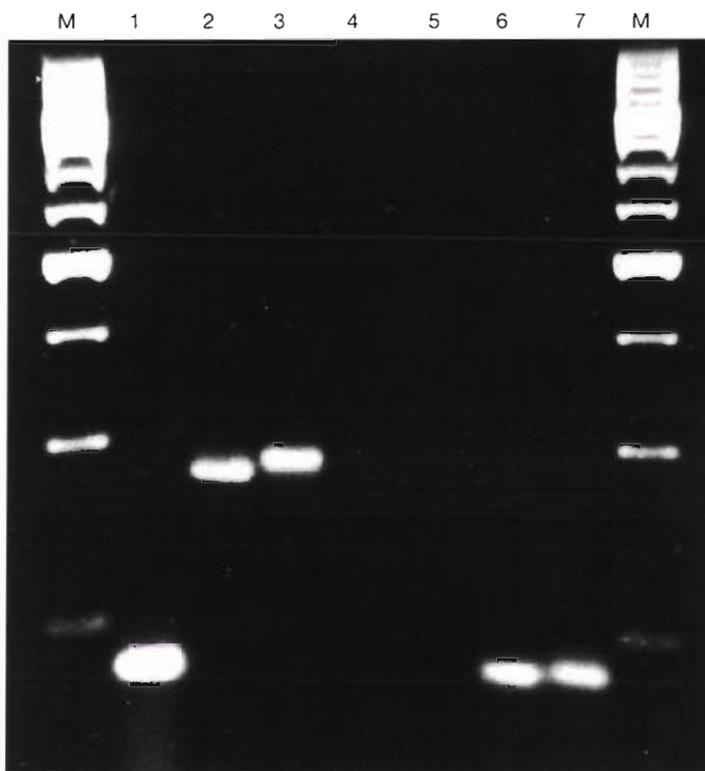


Fig. 4. Agarose gel showing RT-PCR amplification products (10  $\mu$ l) stained by ethidium bromide. DNA marker (M) is a ladder from 250 up to 3000 bp (the steps are 250 bp). Lane (1) is an ISAV positive control (RNA from the kidney of an ISAV challenged Atlantic salmon). RNA from kidney of control and ISA-challenged trout have been used in the RT-PCR in lanes 2–7. Lanes 2 and 3 are MHC-I from 2 trouts (primer set MHC-Ia/MHC-Ib). Lanes 4 and 5 are from control trout and lanes 6 and 7 are from ISAV-challenged trout (primer set FA-3/RA-3)

virus most likely to occur as a co-infection with ISAV in Norway (authors' pers. obs.). Still, when these primers are used for screening for ISA in other countries than Norway, the product should be sequenced to secure that the product is from an ISA virus, although it is not very likely that the primer set would give a segment of equal size if they anneal to some other virus template. The eighth segment of the ISA virus is conserved enough for the FA-3/RA-3 primer set to be used also for diagnosis of ISA in Atlantic salmon from Scotland and the East Coast of Canada (authors' pers. obs.).

Due to difficulties when doing titration to find the number of infective particles (pfu) we had to design a set of different sensitivity tests for the primer sets without this knowledge. In the endpoint titration ( $TCID_{50} = 10^{6.4} \text{ ml}^{-1}$ ) test 37.0 fg viral RNA from the cell culture supernatant could be detected by the RT-PCR (FA-3/RA-3 primers). Transmission electron microscopy of ISA virus-infected cell cultures have shown that a lot of defective particles (non-infective particles judging from the morphology) are also formed during budding from the cell surface (authors' pers. obs.), which means that the number of virus particles containing the genome is higher than the number of infective particles observed when doing the endpoint titration. When the RT-PCR is carried out on trout tissue, the technique is also able to pick out ISA virus RNA from infected cells, i.e. virus RNA that is still inside infected cells and not in virus particles. Infected cells may contain large amounts of ISA virus genome (cf. Koren & Nylund 1997). In addition, neutralising antibodies are produced in the trout (Nylund et al. 1994a), which also influences the ratio between infective and non-infective particles. This probably explains why the RT-PCR is more sensitive than the ASK cells in diagnosis of experimentally infected carrier trout (Table 5).

When as little as 75.0 pg RNA from kidney of ISA-challenged Atlantic salmon was used as template for the RT-PCR, an amplification product could be visualised by ethidium bromide staining (primer set FA-3/RA-3). The RNA was extracted from an ISA-challenged salmon with no clinical signs of ISA and a normal haematocrit value. Other salmon in the same tank showed clinical signs of the disease. Lack of clinical signs in the chosen specimen could be indicative of a lower number of infective particles in the tissue used compared to tissues from salmon with clinical disease. This test differs from the previous one in that most of the RNA is host RNA and not ISA virus genome, which explains the difference in sensitivity. Still, this

Table 5. *Salmo trutta*. Comparison of the results from the screening for ISAV in trout tissues with the help of cell-culture (ASK cells) injection into disease-free salmon (Trans) and RT-PCR. Number of days after challenge of the trout is given in the first column. The column under RT-PCR gives the fraction of ISAV-positive individuals. (+) Positive sample; (-) negative sample

Days	ISAV-challenged trout (ST)			Control trout (KT)	
	ASK cells	Trans	RT-PCR	ASK cells	RT-PCR
	Blood	Kidney		Kidney	
16	-	-	3/11	-	0/9
21	-	-	4/11	-	0/9
54	-	-	(+) <sup>a</sup>	-	0/9
119	-	-	(+) <sup>a</sup>	-	0/9
135	-	+	(+) <sup>a</sup>	-	0/9

<sup>a</sup>No mortality occurred, but the fish sampled after 25 d were found to be positive for ISAV with the help of RT-PCR

test gives an indication of the amount of RNA that is necessary for detecting ISA-positive tissues.

Nishizawa et al. (1994) found that 35 PCR incubation cycles made it possible to detect a quantity of striped jack NNV RNA down to a level of approximately 0.1 fg (the DNA product was about 430 bp), and Wongteerasupaya et al. (1997) showed in an RT-PCR-based detection of yellow-head virus of *Penaeus monodon* that 10.0 fg of purified YHV RNA could be demonstrated. In a study of foot-and-mouth disease virus the detection limit for the RT-PCR (30 to 40 cycles) was determined to be 10<sup>4</sup> template molecules (Niedbalski et al. 1998). The threshold of detection for our primer set (FA-3/RA-3) is slightly higher than the results presented in these studies.

The sensitivity test of the PCR reaction shows that between 300 and 3000 templates (or between 0.22 and 2.2 fg dsDNA) is enough to give a positive result using the described protocol. Oshima et al. (1998) found that 30 PCR cycles were enough to detect a 187 bp DNA fragment from samples containing as little as 10 fg of DNA (which corresponds to approximately 2000 mol of a 3800 bp plasmid). In a study of the PCR detection limit of sporoplasma cells from *Myxobolus cerebralis* using primers against a part (415 bp) of the repetitive 18S rDNA, Andree et al. (1998) were able to detect as little as a single cell. However, the number of copies (which may be very high) of 18S rDNA in a single cell of *M. cerebralis* is not given. Even if contaminating host chromosomal DNA and other tissue contaminants may decrease the sensitivity of our PCR, the threshold of detection for our primer set (FA-3/RA-3) in the PCR seem to be in accordance with the sensitivity presented in the above-mentioned studies.

The RT-PCR performed directly on RNA from kidney of infected trout was the most sensitive method for detection of ISA-carrying trout. Similar results, i.e.

RT-PCR as the most sensitive diagnostic method, have been found in other studies of RNA viruses infecting fish, other vertebrates, and shellfish, i.e. VHSV and IHN (Miller et al. 1998), striped jack nervous necrosis virus (Nishizawa et al. 1994), human coronaviruses, 229E and OC43 (Sizun et al. 1998), and Taura syndrome virus (Nunan et al. 1998). Direct examination of tissues by means of RT-PCR is an effective and powerful assay for detecting ISAV in experimentally infected carrier trout and has advantages over the other methods in speed and sensitivity.

Virus survival in nature requires continuous infection of susceptible individuals or establishment of long-term persistence within infected hosts (Fields et al. 1996). During acute infections, virus is cleared by the host immune response, necessitating rapid transmission or capacity for extra-organismal survival. Persistence of virus within an individual host usually starts with an acute infection that progresses to latent or chronic infection, during which the virus is transmitted periodically to new hosts. Previous transmission trials seem to indicate that acute infections in *Salmo salar* is followed by viral clearance (authors' pers. obs.), while a chronic infection seem to be established in *S. trutta* (Nylund et al. 1995b). This is also confirmed by the present study. There may, however, be individual differences within those 2 species. Salmon that are experimentally infected are usually ISA negative after 135 d; however, in one case a maturing Atlantic salmon (male) was found positive for ISA virus (RT-PCR) several months after challenge. This could indicate that the RT-PCR is not sensitive enough to pick out ISA-carrying salmon, i.e. there are fewer cells infected and fewer particles produced in asymptomatic salmon than in carrier trout. An experimental study of Atlantic salmon surviving an ISA challenge should be performed to establish what percentage of salmon can act as carriers in a population. This study should also give an indication of the importance of stress like sexual maturation (cf. Nylund et al. 1995b) and salmon lice (cf. Nylund et al. 1993, Rolland & Nylund 1998) as a factor that may increase the ISA virus production.

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