Herring (*Clupea harengus*): A host for infectious salmon anemia virus (ISAV).

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Abstract

The emergence of infectious salmon anaemia virus (ISAV) in several countries outside Norway and frequent new outbreaks of the disease within Norway, strongly suggests that there are natural reservoirs for the virus, probably in fish occurring in the coastal waters. Both in Norway and Canada fish farmers have claimed that there could be a possible connection between wild herring (C*lupea harengus*) migrating through fish farms and an outbreak of ISA in the same farms. It has also been claimed that wet feed made from herring could contain the ISA virus and, hence, transmit the disease to salmon (*Salmo salar*). Both these claims are "mythical" in that respect that they are not based on any scientific study or verification that the ISA virus may propagate in herring. Hence, the aim of this study was to challenge herring with the ISA virus, check for virus replication and see if the virus could be transmitted from challenged herring to disease-free Atlantic salmon. With the help of RT-PCR it was shown that the herring became infected with the ISA virus after bath challenge. A drop in haematocrit towards day 20 followed the infection. One salmon that was challenged with filtered homogenate made from ISA challenged herring died. However, most of the salmon survived, but they were positive in the RT-PCR test. It is concluded that the ISA virus is able to propagate in herring and that the herring may be an asymptomatic carrier of the virus.

Introduction

The emergence of infectious salmon anaemia virus (ISAV) in Canada (Mullins et al. 1998), Scotland (Rodger et al. 1998, Rowley et al. 1999), Faeroe Islands, Chile (Kibenge et al. 2001a) and USA (Bouchard et al. 2001) and frequent new outbreaks of the disease in Norway, strongly suggests that there are natural reservoirs for the virus, probably in fish occurring in the coastal waters. The ISA virus has been shown to replicate in Atlantic salmon (*Salmo salar*), trout (*S. trutta*) and *Oncorhynchus* spp., and it can not be excluded that the virus may also replicate in other species (Thorud & Djupvik 1988, Hovland et al. 1994, Nylund et al. 1994, 1995abc, 1997, Nylund & Jakobsen 1995, Kibenge et al. 2001a, Raynard et al. 2001, Snow et al. 2001). Both in Norway and Canada fish farmers have claimed that there could be a possible connection between wild herring (*Clupea harengus*) migrating through fish farms and outbreak of ISA in the same farms. It has also been claimed that wet feed made from herring could contain the ISA virus and, hence, transmit the disease to new farms. Both these claims are "mythical" in that respect that they are not based on any scientific study or verification that the ISA virus may propagate in herring. The aim of this study was therefore to challenge herring with the ISA virus, check for virus replication and see if the virus could be transmitted from challenged herring to disease-free Atlantic salmon. Cell cultures, monoclonal antibodies and polymerase chain reaction (RT-PCR using primers against a sequence from the ISA virus) were used as

	Group	N		Challenge	
		herring	salmon		
Experiment 1	1H-1S	77	5	ISAV bath	Tank 1
	1HC	200	-	HBSS	Tank 2
	CH-CS	20	20	ISAV challenged herring	Tank 3
Experiment 2	2H-2S	43	5	ISAV bath	Tank 1
	2HC	55	-	HBSS	Tank 2
	TS	-	30	Homogenate from ISAV challenged herring	Tank 3

Table 1. An overview of the different experimental groups. H = herring, S = salmon, HC = control herring, CH = ISAV-challenged herring cohabiting with ISAV-free salmon, CS = salmon cohabiting with ISAV-challenged herring, TS = salmon challenged with intraperitoneal injection of filtered homogenate from bath-challenged herring. HBSS = Hanks balanced salt solution. ISAV = infectious salmon anaemia virus.

diagnostic tools for testing the presence of the ISA virus (cf. Devold et al. 2000).

Materials and methods

The herring (*Clupea harengus*) were hatched and cultured at our laboratory using *Rhodomonas, Isochrysis, Brachionus plicatilis* and commercial pellet-food (Kvenseth et al. 1997, Balbuena et al. 2000). The smolts (S*almo salar*) were supplied by a smolt hatchery close to Bergen with no history of ISA. The fish were kept at 10 °C in running seawater. The salmon were acclimatised for 8 days to particle (20 µm) and UV-filtered (Katadyn J1 / P, effect: 50 mWs cm^{-2}) seawater (34‰), kept at 10 °C in 0.15 m^3 tanks (flow rate = 5 l min^{-1}; 60 specimens in each), and fed commercial pellets twice a day.

First challenge

The herring used in the first experiment were 0+ with a mean weight and length of 7.0 g and 10.0 cm, respectively. The salmon smolt weight and length were 123 gram and 22 cm, respectively. The fish, five salmon (1S) and 77 herring (1H), in one tank (0.15 m^3) were challenged for 15 minutes in a bath containing 250 ml sonicated blood, collected from Atlantic salmon during a natural outbreak of ISA (isolate ISA8, cf Devold et al. 2001), diluted in 50 l of sea water (Table 1). After challenge the fish were transferred to running seawater. A control group of 200 herring (1HC) were kept in a separate tank. After 6 days 20 herring (CH) from the group challenged with ISA virus, were transferred to a tank containing 20 disease-free salmon (CS). The experimental period lasted from the 4th November 1997 to the 6th April 1998 (154 days).

Second challenge

The weight and length of the herring (0+) and salmon used in the second experiment were 42 g and 16 cm and 314 g and 31 cm, respectively. The fish came from the same cultured stocks as in the first experiment. The experimental conditions were the same as in the first experiment, but the co-habitation between challenged herring and naïve salmon was not included (Table 1). The bath challenge was repeated with 43 herring (2H) and 5 salmon (2S) in one tank. The ISAV used in this second chal-

lenge was collected in 1998 (isolate ISA36) from infected Atlantic salmon (*Salmo salar*) in a fish farm outside Bergen (cf. Devold et al. 2001). 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 Hank´s balanced salt solution (HBSS) before it was used in the bath challenge. A control group of 55 herring (2HC) was kept in a separate tank. Tissue samples were taken from the heart, liver and kidney of the herring (2H) 26 days after the bath challenge. The tissues were homogenised, filtered sterile, diluted 1:15 in PBS and inoculated on a cell culture (salmon head kidney cells – ASK, Devold et al. 2000). In addition, some of the filtrate was injected intraperitoneal into 30 salmon (TS), 1 ml into each specimen. The experimental period lasted from the 8th April to the 30th May 1998 (53 days).

Several tissue samples (kidney, spleen, heart and skin) were collected from the herring and salmon in both experiments. The tissues were frozen in liquid nitrogen and stored at -80°C for later use (RT-PCR and cell-culture).

The following measurements were made of the fish sampled in both challenge experiments: weight, length, clinical signs of disease and blood (for haematocrit measurements and blood smears for study of blood cell morphology). The haematocrit values did not have a normal probability distribution; hence, a nonparametric test (Mann-Whitney *U*-test) was used to compare different groups after challenge. The groups were considered significantly different when $P < 0.05$.

A few selected fish were examined bacteriologically by inoculation of kidney tissue on blood agar plates which were incubated at 20 °C for 6 days, and on blood agar plates containing 2 % NaCl which were incubated for 6 days at 15 °C.

Cell culture

The challenged herring were screened, by cell culture (ASK cells), for the presence of ISAV 20 days after challenge (Devold et al. 2000). Kidney tissue was homogenised and cleared by centrifugation before filtration (0.2 mm). The homogenates were diluted 1:100 in PBS and incubated for 3 hours at 15°C in cell culture flasks with a mono-layer of ASK cells. The inoculum was then removed and replaced by supplemented L-15 medium with 5% FCS. The cells were incubated at 15°C for 12 days or until cytopathic effect (CPE) could be observed. The medium from cells considered negative after 12 days was used as inoculum in a passage to new cells. The samples were passed three times before they were considered negative. A negative and a positive control cell culture were always included. Homogenates from the original challenges were used as positive controls.

The cell cultures were also stained with a monoclonal antibody against the ISAV. Before staining the cells were washed three times in PBS pH 7.4 plus 0.1% Tween-20 and fixed in 80% acetone for 20 minutes. After complete drying the cells were blocked with 5% milk powder in PBS for 30 min at room temperature. The primary mouse anti-ISA MAb (3H6F8) diluted 1:200 was inoculated at 4°C over night. The cells were washed 3 times with PBS Tween before the secondary antibody FITC-labelled goat anti-mouse Ig diluted 1:100 was incubated at 20°C for 60 minutes. The cell nucleus was stained with propidium iodide (Sigma P-4170, 10mg/ml) for 1 minute and washed with PBS Tween 3 times. The

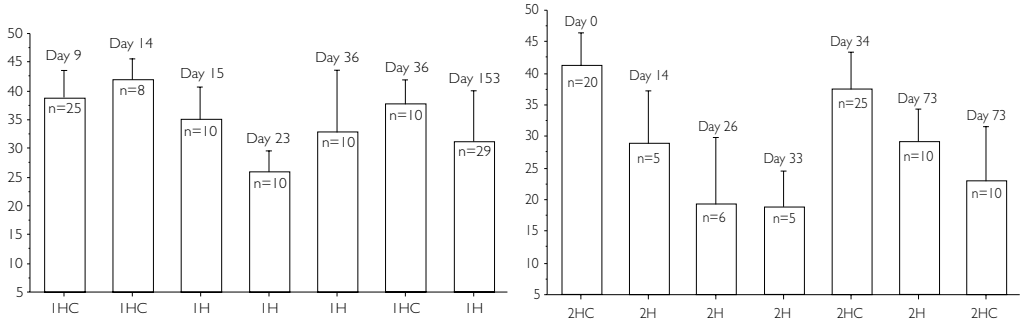

Figure 1. Mean haematocrit values of control and ISAV-challenged herring at different sampling dates after challenge. HC = control herring, H = ISAV-challenged herring. A = Experiment 1, B = experiment 2.

stained cell cultures were checked at x1000 magnification on a Zeiss fluorescence microscope (Axioskop).

RT-PCR diagnostics

The RNA was extracted from kidney tissue using Trizol reagent (Life Technologies) according to standard protocols (Devold et al. 2000). Reverse transcription was performed using MMLV-RT (Promega) with random hexamers as primers (Devold et al. 2000). Subsequently, the single stranded cDNA served as template in PCR using Taq DNA polymerase according to recommended conditions (Pharmacia). The following diagnostic primers were used: FA-3: 5´- GAA GAG TCA GGA TGC CAA GAC G - 3´ and RA-3: 5´- GAA GTC GAT GAT CTG CAG CGA - 3´. The primers give a segment of 221 bp (Devold et al. 2000). The reaction cycle was: Denaturation at 94 °C for 3 minutes and amplification performed with 35 cycles of 94°C for 30 seconds, 59 °C for 45 seconds, and 72 °C for 90 seconds. The reaction was terminated by an extension for 10 min at 72 °C. The tubes were then held at 4 °C. Aliquots (10µl) of the PCR reaction mixture were run by electrophoresis on a 1% agarose 1 x TBE gel containing 1 µl ethidium bromide and photographed under UV transillumination.

The PCR products were purified on Qiaquick PCR Purification columns (Qiagen) and then sequenced using the BigDye Terminator Sequencing kit. The products were run in an ABI 377 DNA analyzer (PE Biosystems). The sequence data were assembled with the help of Vector NTI software (InforMax, Inc.) and the GenBank searches were done with BLAST (2.0). The Vector NTI Suite software package (InforMax) was used for the multiple alignments of the nucleotide sequences.

Results

The Atlantic salmon (*Salmo salar*) that were used as positive controls during the bath challenges in the two experiments died with clinical signs of ISA, with the exception of two salmon that survived in the first experiment. The dead salmon were confirmed ISAV positive with RT-PCR using primers FA-3 and RA-3. One of the two survivors was also ISAV positive by RT-PCR.

Only one herring died (29 days after challenge) in group 1H from the first experiment. The herring that died had a low haematocrit (= 10%), but no other clinical signs of disease. The mean haematocrit values of the herring dropped after challenge and were as low as

26.5% (S.D. = 3.37; range = 20 – 32) 23 days after challenge in experiment 1 (Figure 1A). The haematocrit values were not significantly ($p > 0.05$) different from the controls 15, 36 and 153 days after challenge.

Five herring (1H) collected 23, 36 and 42 days after bath challenge were screened for ISAV by RT-PCR. Of these 4/5, 4/5 and 3/5 were positive for ISAV. Three herring died in group CH one day after transfer to a new tank and seven days after challenge. These had pale gills and empty guts. None of the salmon (group CS) that co-habited with the ISA challenged herring (group CH) died during the experimental period. All the salmon in group CS were screened for the presence of ISAV by RT-PCR, but were negative. There was no mortality in the herring control group 1HC during the experimental period. It was not possible to culture ISAV from bath challenged herring (1H) or from salmon (CS) cohabiting with ISAV–challenged herring.

The herring (2H) in the second experiment had a significant drop in the haematocrit values 26 and 33 days after challenge compared to the control group (2HC). The mean haematocrit values at these sampling dates were 20% and 19%, respectively (Figure 1B). The mortality (N = 14, i.e. 32%) of herring in the second experiment (2H) was fairly high during the first 10 days after challenge, but no mortality occured in the control group (2HC). The mortality started six days after challenge and flattened out after 10 days. A total of 17 herring died during the experimental period and the last died 24 days after challenge. No bacteria were isolated from the herring. During the first 42 days 70% of the herring (2H) were found positive for the ISA virus using RT-PCR, but it was not possible to culture the virus from challenged herring (2H). No herring were found positive later than 42 days after challenge. All salmon (TS) that were intraperitoneally injected with filtered homogenate from bath challenged herring were RT-PCR positive for ISAV and one salmon died showing clinical signs of ISA.

Discussion

The increasing numbers of new outbreaks of ISA in Norway during the last few years is a source of much concern for fish farmers and the veterinary service. It was believed that the traditional hygienic measurements that were implemented during the massive outbreak in the years between 1988 and 1992 could keep the disease under control (Håstein et al. 1999). However, since 1994, a year without any new outbreaks of ISA in Norway, there has been a slow but steady increase of new infected salmon farms. Several reasons are given to explain this development; increased number of salmon farms, few or no restrictions on transportation of salmon in well-boats, more reliable reports on new outbreaks of ISA, better ISA diagnostics and other factors connected to salmon farming. It has also been claimed that vaccination against other salmon diseases, using oil-vaccines, may give a non-specific protection against the ISAV resulting in asymptomatic carriers. Presence of carriers within the salmon farming industry would of course increase the possibility of spreading the virus. Presence of a European ISAV isolate in Canada (Kibenge et al. 2001b, Ritchie et al. 2001) is a strong indication that human activity may move the virus between farming regions. This is also supported by other studies (Jarp & Karlsen 1997, Devold et al.

2001, Murray *et al*. 2002).

In the hunt for explanations little attention has been focused on the possibility and importance of natural reservoirs in Norwegian waters. Outbreaks of ISA in other countries (Canada, USA, Scotland, Faeroe Islands, and Chile), that cannot be connected to ISA in Norway, show that transmission from natural reservoirs to farmed salmon is not a freak occurrence. Presence of ISAV in local wild populations of trout and salmon in Scotland suggests that these salmonids in Norway (which has larger populations of salmon and trout) may constitute important ISAV reservoirs (Raynard et al. 2001). A thorough screening of wild salmonids in Norway remains to be carried out.

In addition to salmonids, other fish species may carry the ISA virus. Different wrasse species have been challenged and found to be non-susceptible to ISAV (Kvenseth *et al*. 1998), while other salmonids, *Oncorhynchus* spp. and *Salvelinus alpinus*, may function as hosts for the virus (Nylund *et al*. 1994, 1995abc, 1997, Nylund & Jakobsen 1995, Kibenge et al. 2001a, Raynard *et al*. 2001, Snow *et al*. 2001). Herring, *Clupea harengus*, are more closely related to salmonids than wrasse species, which, together with the "mythical" claims that this species may be responsible for transmission of ISAV to farmed salmon, made it a candidate for a closer study.

Positive RT-PCR as late as 42 days after bath challenge shows that ISAV is able to enter and propagate in herring. It was not possible to culture ISAV from herring in ASK cells and cohabitation with infected herring and disease-free salmon did not result in transmission of the virus to the salmon. However, virus isolated from infected herring (filtered homogenates) was able to infect and kill salmon. It should also be pointed out that the cohabitation experiment was performed in November-December, i.e. a time of the year when it has proved difficult to infect and kill salmon with ISAV (pers.obs.). Hence, it cannot be excluded that ISAV-infected herring may transmit the virus to ISAV-free salmon when a school of herring passes through net pens. It should also be safe to assume, based on the present study, that wet feed made from herring could transmit ISAV to salmon unless treated so as to kill the virus. The latter could easily be controlled by the salmon farmers, while the only way to prevent transmission from wild herring would be to use closed net pens or move the salmon production to land-based tanks. The last option is not likely to be chosen at present, which means that in certain "hot spots", areas with frequent outbreaks of ISA, vaccination against ISAV should be considered. Vaccination of salmon would be even more necessary if it turns out that a large portion of the wild local populations of trout and salmon are carriers of ISAV.

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