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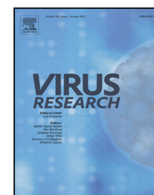

Infectious salmon anemia virus (ISAV) replication is transiently inhibited by Atlantic salmon type I interferon in cell culture☆

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ABSTRACT

Infectious salmon anemia virus (ISAV) is a piscine orthomyxovirus, which causes multisystemic disease in farmed Atlantic salmon that may result in large losses. Previous work has suggested that ISAV is able to resist the antiviral state induced in cells by type I interferon (IFN). These studies were, however, mainly based on cytopathic effect (CPE) reduction assays. Here we have investigated the antiviral activity of Atlantic salmon IFNa1, IFNb and IFNc against ISAV using quantitative PCR (qPCR) of segment 6, Western blot analysis of ISAV proteins and viral yield reduction assays, in addition to CPE reduction assays. Antiviral effects of IFNs were tested against the high virulent strain ISAV4 and the low virulent strain ISAV7 both at the optimum growth temperature 15 °C and at 20 °C. As expected, IFNa1 showed little protection against CPE development in cells after infection with both strains at 15 °C. However, the qPCR and Western blot analysis clearly showed strong inhibition of replication of the virus strains by IFNa1 between 24 and 72 h after infection. The inhibitory effect declined four to five days post-infection, which explains the low protection against CPE development 7–10 days later. At 20 °C, IFNa1 showed strong protection against CPE development, probably due to slower virus growth. IFNc showed similar antiviral activity as IFNa1 against ISAV4 while IFNb showed lower activity. There were observed differences between ISAV4 and ISAV7 both with respect inhibition by IFNa1 and ability to induce the two IFN-inducible antiviral effector proteins, Mx and ISG15, which may be related to differences in virulence properties and/or adaption to growth in cell culture.

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1. Introduction

Infectious salmon anemia virus (ISAV) is a piscine orthomyxovirus of the *Isavirus* genus, which causes a multisystemic disease in farmed Atlantic salmon (*Salmo salar* L.) (Falk et al., 1997; Krossøy et al., 1999). Like the influenza A virus, ISAV is enveloped and has a genome composed of eight linear, single-stranded RNA segments of negative polarity (Mjaaland et al., 1997). The eight segments give rise to a total of ten proteins where segment 6 (s6) encodes the surface glycoprotein haemagglutinin–esterase (HE) (Cottet et al., 2011; Krossøy et al., 2001; Rimstad et al., 2001).

Type I interferons (IFNs) play a crucial role in the first line of defense against viruses in vertebrates. The type I IFNs are cytokines with antiviral activity, which are produced and secreted after recognition of viral nucleic acid by host pathogen recognition receptors such as TLR3, TLR7 and RIG-I-like receptors. Secreted IFN binds and signals through the IFN-receptor (IFNAR) expressed on the surface of host cell, and activation of IFNAR leads to upregulation of hundreds of interferon stimulated genes (ISGs). Many ISG-products, such as ISG15 and Mx, have direct antiviral effects and collectively induce an antiviral state in the cell (Sadler and Williams, 2008; Takeuchi and Akira, 2007). In Atlantic salmon, four different subtypes of type I IFN have been reported, and these are encoded by one to several genes: IFNa (three genes), IFNb (four genes), IFNc (five genes) and IFNd (one gene) (Sun et al., 2009; Svingerud et al., 2012). IFNa1, IFNb and IFNc have been confirmed to have antiviral activity against IPNV and to induce several ISGs including Mx, ISG15, ISG58, viperin and protein kinase R (PKR), whereas IFNd does not display antiviral activity against IPNV and does not induce Mx protein (Svingerud et al., 2012). Atlantic salmon IFNa1 has previously also been demonstrated to have antiviral activity of against salmonid alphavirus 3 (Xu et al., 2010). Pretreatment of Atlantic salmon TO cells with recombinant IFNa1 did, however, not inhibit ISAV-induced CPE, whereas a modest reduction in CPE was observed in SHK-1 cells (Jensen and Robertsen, 2002; Kileng et al., 2007). Intraperitoneal injection of

Abbreviations: ASK, Atlantic salmon kidney cells; CPE, cytopathic effect; HE, haemagglutinin–esterase; IFN, interferon; ISAV, infectious salmon anemia virus; ISG, interferon stimulated gene; NP, nucleoprotein; PKR, protein kinase R.

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Atlantic salmon with the potent inducer of type I IFNs, polyinosinic polycytidylic acid, has however been shown to reduce the cumulative mortality of ISAV-infected fish compared to control fish (Jensen et al., 2002).

Two ISAV strains were used in the present study, ISAV4 (Glesvaer/2/90) and ISAV7 (ISAV96/09/1729). These strains have been reported as high- and low-virulent, respectively, based on the mortality obtained in challenge experiments with Atlantic salmon (Mjaaland et al., 2005). Both viruses have a lysine in position 266 of the fusion protein and close to identical HE sequences (Markussen et al., 2008), which are two traits that have been linked to virulence (Markussen et al., 2008; Mjaaland et al., 2005; Plarre et al., 2012). The ability to antagonize the IFN system is known to play a part in virulence in other viral strains, such as Influenza A virus (Jackson et al., 2008; Li et al., 2006b), but if this also applies to ISAV is at the present not known. However, two proteins named s7ORF1 and s8ORF2, have been shown to inhibit activation of the salmon IFNa1 promoter, and the rainbow trout Mx promoter (only s7ORF1) (García-Rosado et al., 2008; McBeath et al., 2006). s7ORF1 and s8ORF2 from ISAV4 differ from those of ISAV7 by several amino acids (Markussen et al., 2008), but whether these sequence differences can explain difference in strain virulence is unknown.

The main objective of the present paper was to establish if IFNa1 could induce antiviral activity against ISAV in salmon cells by the use of several different antiviral assays. We also wanted to test if IFNa1, IFNb and IFNc showed differences in antiviral activity against ISAV. Finally, we studied whether ISAV4 and ISAV7 showed differences in sensitivity to the antiviral state induced by IFNa1 and if the two viruses showed differences in ability to induce the antiviral genes Mx and ISG15 in salmon cells.

2. Materials and methods

2.1. Cells and viruses

ASK cells derived from Atlantic salmon head kidney (Devold et al., 2000) were purchased from American Type Culture Collection. ASK and TO cells (Wergeland and Jakobsen, 2001) were grown at 20 °C in L-15 medium (Gibco) containing 1× MEM Non-Essential Amino Acid Solution (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin and 15% (ASK) or 8% (TO) FBS Superior (Biochrom AG).

The ISAV4 (Glesvaer/2/90) and ISAV7 (ISAV96/09/1729) isolates, which originate from ISA outbreaks in Norway (Mjaaland et al., 2005), were obtained from the Norwegian Veterinary Institute, Oslo. Virus propagation was performed in ASK cells, whereas viral titers were determined by endpoint titration in TO cells. Infection was carried out by absorbing the viral suspension to the cells in serum-free medium for 3 h before removing the medium and adding L-15 containing 2.5% serum. Infection and post-infection incubation were carried out at 15 °C in most experiments, with the exception of the CPE reduction assays, which were performed both at 15 °C and 20 °C, and the experiments comparing the antiviral effect of IFNa1, IFNb and IFNc, which were carried out only at 20 °C. Unless otherwise stated a multiplicity of infection (MOI) of 1 was applied.

2.2. Interferons

Recombinant Atlantic salmon IFNa1 (GenBank accession no. DQ354152.1), IFNb (GenBank accession no. JX524152) and IFNc (GenBank accession no. JX524153) were produced by transfection of HEK-293 cells with IFN expression plasmids as described (Svingerud et al., 2012). The IFN yields were estimated to be 850,000, 5700, 8,200,000 U/ml, respectively, where 1 unit (U) of IFN activity was defined as the reciprocal dilution of the supernatant causing 50% reduction in IPNV-induced CPE in ASK cells. In the various experiments, the IFNs were diluted in L-15 medium containing 15% FBS Superior and supplements before addition to cells. IFNb shows the highest amino acid sequence identity (99.5%) to the previously described IFNb2 sequence, while the IFNc sequence is most similar to IFNc1 (99.5%) (Sun et al., 2009; Svingerud et al., 2012).

2.3. Antibodies

The rabbit anti-ISAV HE antibody was generous gift from Dr. Anita Müller (University of Tromsø) (Müller et al., 2008). Mouse anti-ISAV nucleoprotein antibody (clone 2C2/H4) was from Immunological and biochemical testsystems GmbH. Rabbit anti-salmon Mx1 antibody was made against recombinant Atlantic salmon Mx1 protein in our laboratory by Dr. Hilde Hansen. The rabbit anti-ISG15 is described in Røkenes et al. (2007). The rabbit anti-actin antibody was obtained from Sigma–Aldrich, whereas the secondary antibodies, goat anti-rabbit-horseradish peroxidase and goat anti-mouse-horseradish peroxidase were from Santa Cruz Biotechnology.

2.4. CPE reduction assay

The CPE reduction assay was performed as described earlier with some modifications (Berg et al., 2009). Briefly, ASK cells were seeded in 96-well plates at 25,000 cells per well. The following day, the IFNa1 batch was diluted in L-15 medium containing 15% FBS and supplements, and various concentrations (1000, 500, 200, 50 and 5 U/ml) were added onto the cells at 100 μl per well ($n = 4$). After 24 h, the IFNa1-containing medium was removed and the cells were infected with ISAV4 or ISAV7 (Section 2.1). Untreated control cells, infected and uninfected, were included. When complete CPE was observed in untreated control cells, the cells were stained with crystal violet, dissolved in 50% ethanol containing 0.05 M sodium citrate and the absorbance was read at 550 nm. To compare the antiviral effect of IFNa1, IFNb and IFNc, a similar experiment was performed using ISAV4 and 500, 50 and 5 U/ml of each IFN.

2.5. Inhibition of ISAV replication by IFN, and induction of Mx by ISAV4 and ISAV7 (qPCR)

Replication of ISAV4 and ISAV7, and the inhibiting effect of IFNa1 were determined by studying the levels of ISAV s6 RNA using qPCR. ASK cells were seeded in 24-well plates at 1.5×10^5 cells per well and treated with 1000 U/ml of IFNa1 in 1 ml of medium the following day or mock-treated (control). The cells were infected with ISAV4 or ISAV7 after 24 h of treatment by replacing the medium with medium containing virus. Cells from triplicate wells were harvested at 4, 24, 72 and 120 h post-inoculation for RNA isolation and qPCR analyses (Section 2.6). The mock-treated samples from the 72 h time point were also used to determine the induction of Mx gene expression by ISAV4 and ISAV7. To compare the antiviral effect of IFNa1, IFNb and IFNc, ASK cells were treated with 1000 U/ml of each IFN or left untreated ($n = 3$). After 24 h the cells were infected by ISAV4 as described above. Cells were harvested at 72 h p.i. for RNA isolation and qPCR analysis.

2.6. RNA isolation, cDNA synthesis and relative qPCR

RNeasy Mini Kit from Qiagen was used for RNA isolation following the manufacturer's recommendation. Cells were lysed in 350 μl RLT buffer containing 10 μl/ml 2-mercapto-ethanol at the indicated time points (Section 2.5), and the samples were homogenized using a 0.9 mm needle connected to a syringe. cDNA synthesis was performed with the QuantiTect Rev. Transcription Kit (Qiagen)

Table 1
Primers used for qPCR.

Primer name	Sequence
18S rRNA forward	5'-TGTGCCGCTAGAGGTGAATT-3'
18S rRNA reverse	5'-GCAAATGCTTTCGCTTTCG-3'
ISAV-s6 forward	5'-GAAGCAGATGAGTGGAAGGTTTC-3'
ISAV-s6 reverse	5'-CCGTCAGTGCAGTCATTGGT-3'
Mx- forward	5'-TGCAACCACAGAGGCTTTGAA-3'
Mx-reverse	5'-GGCTTGGTCAGGATGCCTAAT-3'

using 150 ng total RNA. PCR was performed in a 15 μl reaction mixture containing 6.1 μl 1:10 dilution of cDNA (except for detection of 18S rRNA, where a 1:1000 dilution was applied), 7.5 μl Fast SYBR Green Master Mix (Applied Biosystems) and 230 nM of forward and reverse primers (Table 1). The qPCR was run in triplicate wells on a 7500 Fast Real Time PCR system (Applied Biosystems), and the PCR program was as follows: 1 cycle of 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. A melt curve stage was included to confirm the absence of primer–dimers and nonspecific products. The relative expression of ISAV s6 and Mx was normalized against the expression of 18S rRNA as described (Kileng et al., 2007). Fold-increase of s6 was calculated relative to the ISAV4-infected IFNa1-treated 4 h time point (set to 1), while fold-upregulation of Mx was calculated relative to uninfected untreated cells. In the experiments comparing the effect of IFNa1, IFNb and IFNc, the upregulation of s6 was calculated relative to the untreated infected control cells.

2.7. Viral yield reduction assay

ASK cells were seeded in 24-well plates at 1.5×10^5 cells per well and treated with 1000 U/ml in 1 ml medium of IFNa1 or medium (control) the following day. The IFN-containing medium was removed after 24 h and the cells were infected with ISAV4 or ISAV7. Supernatants from triplicate wells were harvested for virus titration 72 h post-infection, which were determined by end-point titration in TO cells using the $TCID_{50}$ method (Reed and Muench, 1938). The antiviral effect of IFNa1, IFNb and IFNc against ISAV4 was compared in a similar experiment using 1000 U/ml of each IFN.

2.8. Western blot analysis

To study the inhibitory effect of IFNa1 against ISAV4 and ISAV7 HE protein production, and to determine the effect of virus infection on Mx expression, ASK cells were seeded in 24-well plates at 1.5×10^5 per well, and treated with 1000 U/ml IFNa1 or medium (controls). The cells were infected with ISAV4 or ISAV7 24 h later, and harvested 24, 48, 72, 96 and 120 h post-infection in 70 μl SDS sample buffer. SDS-PAGE was performed using 10 μl of each sample on a 4–12% NuPAGE Bis–Tris Gel (Invitrogen). The proteins were blotted onto PVDF membranes, blocked with 5% dry milk and incubated overnight with anti-HE antibody (1:2000) and anti-Mx antibody (1:3000) at 4 °C followed by 1 h incubation with the secondary antibody (goat-anti rabbit HRP) at a dilution of 1: 20,000. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate from Pierce, and images were captured on a Kodak image station 4000MM pro from Carestream Health, Inc. To detect actin, the membranes were stripped before being incubated with the anti-actin antibody (1:1000) overnight (4 °C) followed by re-incubation with secondary antibody. Stripping of the membranes was performed by 5 min incubation with dH_2O, 10 min in 0.2 M NaOH, followed by 5 min wash in dH_2O.

The inhibitory effects of IFNa1, IFNb and IFNc on production of ISAV4 HE protein and ISAV4 nucleoprotein (NP) were compared using 1000 U/ml of each IFN in an otherwise similar experiment. A dilution of 1:2000 of the anti-NP antibody was applied to detect NP. Both goat-anti rabbit HRP and goat-anti mouse HRP were used as secondary antibodies, at a dilution of 1:20,000 and 1:10,000 respectively. Virus inoculation and the following incubation were carried out at 20 °C, and the samples were harvested only at 72 h p.i. The net intensity of the NP- and actin bands from triplicate lanes was measured by Carestream Molecular Imaging software (Version 5.0), and the NP/actin ratio was calculated. The inhibitory effect of IFNa1, IFNb and IFNc on ISAV NP production was presented as the percent intensity of the NP/actin ratio in the treated cells relative to the untreated, infected control samples.

The expression of Mx and ISG15 in ISAV4 and ISAV7-infected ASK cells were determined by Western blot analyses. ASK cells were seeded in 24-well plates at 1.5×10^5 per well and infected with ISAV4 or ISAV7 at a MOI of 5 the following day. A high virus titer was used to ensure that all cells were infected. After 24 h, cells were harvested and the presence of Mx, ISAV NP and actin was determined as described above. In this experiment, the anti-NP antibody was used at a dilution of 1:500. To detect ISG15, the membrane was stripped before proceeding to the primary antibody incubation using a dilution of 1:20,000. The net intensity of Mx, ISG15 and actin bands run in triplicate lanes were measured by Carestream Molecular Imaging software, and the ratio of NP/actin- and ISG15/actin was calculated.

2.9. Statistical analyses

Unpaired Student's *t*-test was used to calculate statistics.

3. Results

3.1. Effect of IFNa1 pretreatment on ISAV4 and ISAV7-induced CPE

The CPE reduction assay, which is a widely used method to determine antiviral activity of IFNs, was used to measure the antiviral activity of IFNa1 against the high virulent strain ISAV4 and the low virulent strain ISAV7 in ASK cells. Stimulation with IFN was carried out at 20 °C, while infections were carried out both at 15 °C, which is the optimal growth temperature of the virus, and at 20 °C where the virus replicates more slowly (Falk et al., 1997). Complete virus-induced CPE was observed in the untreated control cells 8 days after infection for the experiments performed at 15 °C and 12 days after infection for the experiments performed at 20 °C. As seen in Fig. 1A and B, pretreatment of ASK cells with IFNa1 induced a slight protection against ISAV4 (8–9%) and ISAV7-induced CPE (11–17%) at 15 °C. A much higher level of protection was observed at 20 °C (Fig. 1C and D), which is most probably due to slower replication of the virus at this temperature (Falk et al., 1997). In contrast to that observed at 15 °C, the replication of ISAV4 was more easily inhibited by IFNa1 compared to ISAV7 when infections were carried out at 20 °C. Some of the cells in the wells infected with ISAV4 alone did not reach complete lysis at the termination of the assay at 20 °C, while all the cells infected with ISAV7 were completely lysed, and the higher inhibition of ISAV4-induced lysis might thus be caused by a slower replication of ISAV4 than ISAV7 at this temperature.

3.2. Effect of IFNa1 pretreatment on replication of ISAV4 and ISAV7

The inhibitory effects of IFNa1 pretreatment on the replication of ISAV4 and ISAV7 at 15 °C were studied in a qPCR time course study of s6 (Fig. 2A and B). The s6 sequences of ISAV4 and ISAV7 are highly similar (Markussen et al., 2008), and the primer pairs were designed to span areas with 100% match. RNA was harvested from IFNa1-treated and untreated ISAV-infected ASK cells at 4, 24, 72 and 120 h post infection (p.i.), and the fold increase of s6 RNA was for all the samples calculated relative to the s6 RNA level in the ISAV4-infected IFNa1-treated cells

at the 4 h p.i. The s6 RNA levels in the 4 h p.i. samples from both the untreated ISAV4 and the untreated ISAV7-infected cells were 1.6-fold higher than this calibration sample, which confirms that comparable amounts of ISAV4 and ISAV7 had infected the cells.

The two strains showed a similar replication rate with a fold increase of s6 RNA of 118,000 (ISAV4) and 126,000 (ISAV7) at 120 h p.i. in untreated cells (Fig. 2A and B). Pretreatment of cells with IFNa1 inhibited the replication of both viruses, with a fold-reduction of 1.4–8.4 at the various time points. The antiviral effect of IFNa1 was most prominent at 24 and 72 h p.i., and the fold-difference between the treated and untreated cells was declining for both strains at 120 h p.i. (Fig. 2A and B). The replication of ISAV7 was, however, slightly more inhibited by IFNa1 at 120 h p.i. compared to that of ISAV4 (2.4-fold *versus* 1.3-fold reduction, respectively).

The effect of IFNa1 pretreatment on replication of ISAV4 and ISAV7 was also determined at the translational level by Western blot analysis of ISAV HE protein in IFN-treated and untreated cells (Fig. 2C and D). In the untreated samples, ISAV4 and ISAV7 HE were detectable from 48 h and displayed strong expression from 72 h. For both strains, IFNa1 strongly inhibited HE production at 48 and 72 h p.i., but the viral protein was increasing at 96 h p.i. and showed a high expression at 120 h p.i.

The expression of host antiviral Mx protein was also analyzed in the same experiment. IFNa1 induced expression of Mx in ASK cells already at 24 h post-treatment, and a high Mx expression was seen throughout the 120 h experimental period. Interestingly, ISAV7 induced increasing Mx protein levels at 24, 48 and 72 h p.i. in untreated cells (Fig. 2D), whereas little Mx was observed in the ISAV4-infected cells at any of the time points (Fig. 2C).

To measure the effect of IFNa1 on the viral yields, the medium supernatants from the 72 h qPCR samples were collected and the viral titers were determined by the $TCID_{50}$ method. As illustrated in Fig. 2E and F, pretreatment of cells with IFNa1 caused a 37-fold and 12-fold reduction in viral yields of ISAV4 and ISAV7, respectively.

3.3. Comparison of the antiviral activity of IFNa1, IFNb and IFNc against ISAV4

The antiviral activity of IFNa1, IFNb and IFNc against ISAV4 was compared by four different methods: CPE reduction assay, qPCR of s6 RNA, Western blot analysis of HE and NP, and reduction in viral yields (Fig. 3). Due to the low antiviral activity of IFNa1 against ISAV in the CPE reduction assay at 15 °C (Fig. 1A), virus infected cells were incubated at 20 °C in these experiments. For the qPCR and Western blot analyses, ASK cells were pretreated with IFN for 24 h prior to infection with ISAV4, and the samples were harvested at 72 h p.i. The CPE reduction assay was terminated when control cells reached complete CPE.

Fig. 3A shows that pretreatment of ASK cells with all three IFNs inhibited ISAV4-induced CPE. IFNa1 and IFNc showed similar antiviral potency, inducing 75–80% cell survival at the highest doses, whereas the IFNb-induced cell survival was 25–30 percentage points lower.

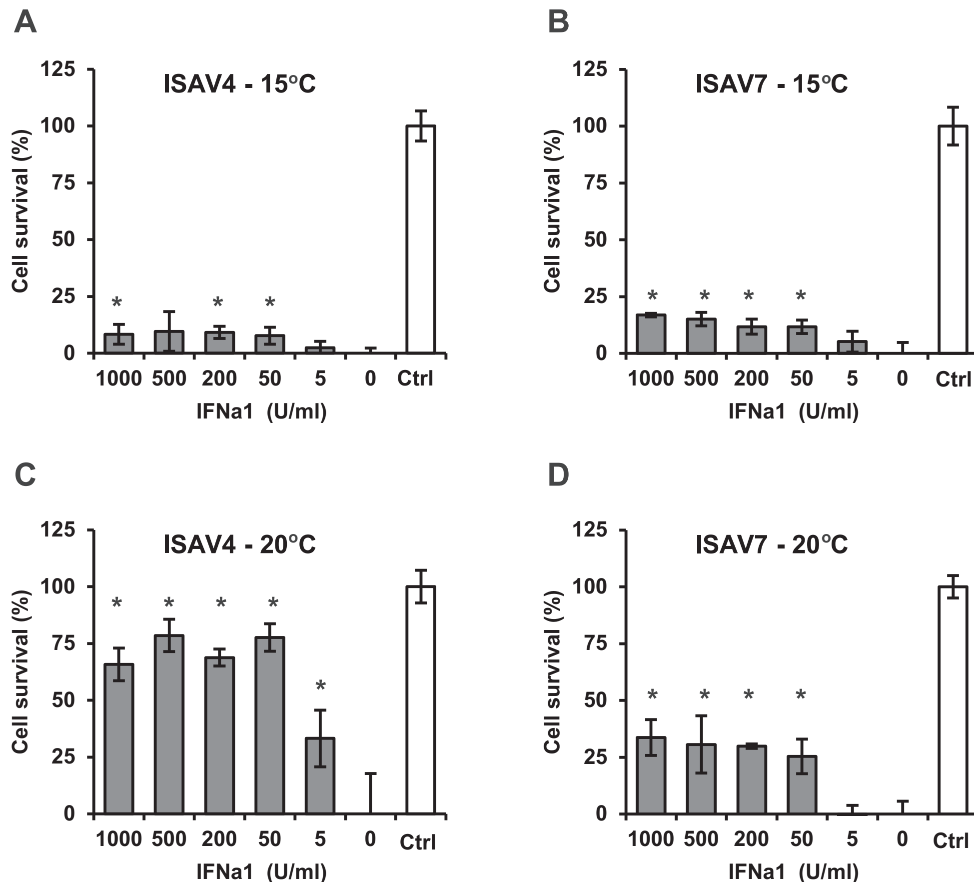

Fig. 1. Cytopathic effect reduction assay of IFNa1 treated ASK cells infected with ISAV4 or ISAV7. ASK cells were treated with various concentrations of IFNa1 and infected with ISAV4 (A and C) or ISAV7 (B and D) 24 h later at a MOI of 1. The virus inoculation and subsequent incubation were carried out at 15 °C (A and B) and at 20 °C (C and D). When untreated control cells showed complete CPE, the cells were stained with crystal violet, and the OD was read at 550 nm. Percentage cell survival was calculated relative to untreated infected cells and untreated uninfected cells. The mean value ± SD from four parallel wells is given. *Values are significantly higher than infected controls cells ($p < 0.05$). Gray bars: virus infected cells; white bars: uninfected control cells.

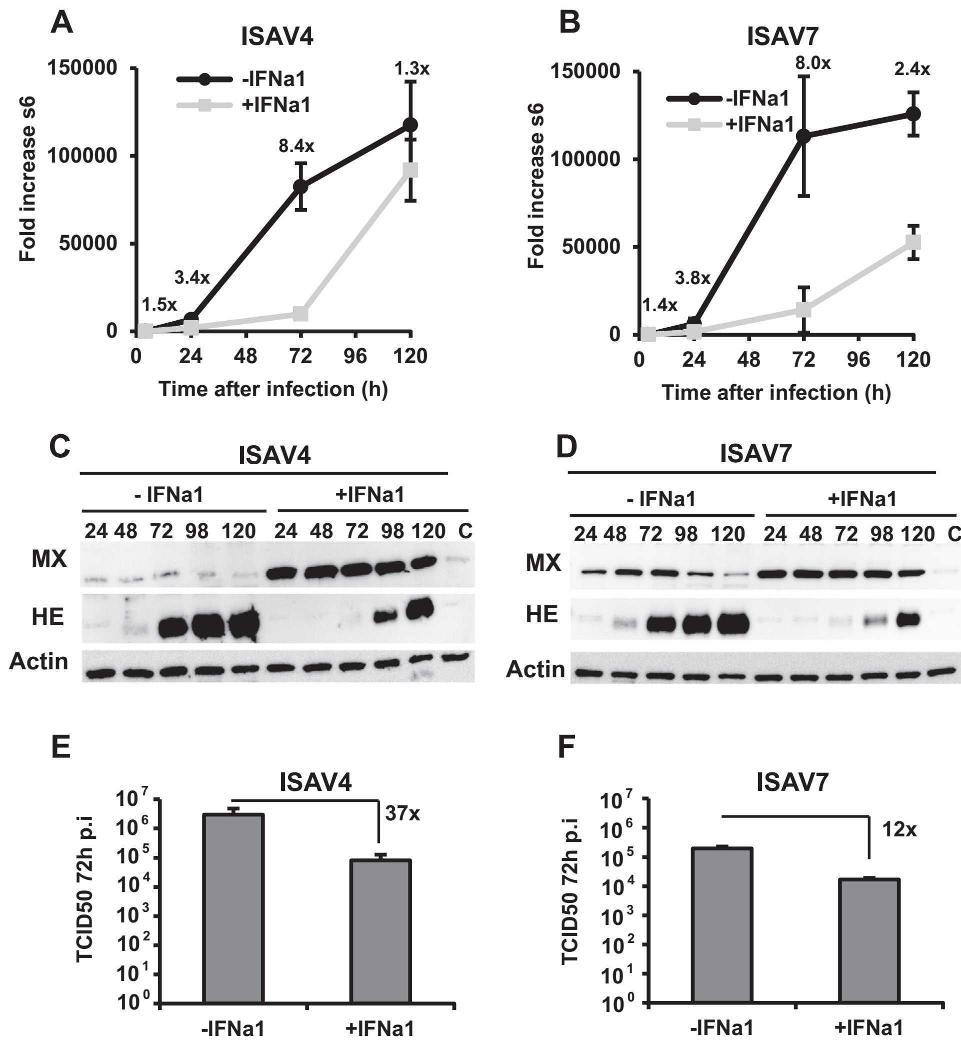

Fig. 2. IFNa1-induced inhibition of ISAV4 and ISAV7. ASK cells were treated with 1000 U/ml IFNa1 or left untreated, and infected with ISAV4 or ISAV7 24 h later (MOI 1). RNA levels of ISAV4 (A) and ISAV7 (B) s6 were determined 4, 24, 72 and 120 h p.i. by qPCR. The expression was normalized against 18S rRNA and the fold upregulation of both strains was calculated relative to the s6 transcript levels in the IFNa1-treated ISAV4-infected cells at 4 h p.i. The mean values from triplicate wells ±SD are given, and the numbers above the lines represent the fold difference between IFNa1-treated and untreated cells. For Western blot analyses, ASK cells were left untreated or pretreated with 1000 U/ml IFNa1 24 h prior to infection with (C) ISAV4 or (D) ISAV7 (MOI 1). Samples were harvested at 24, 48, 72, 96 and 120 h post infection, and the expression of Mx, actin and ISAV HE was detected. C; control samples (untreated uninfected cells). The supernatants from the 72 h qPCR time points (panel A and B) were harvested and viral titers of ISAV4 (E) and ISAV7 (F) were assessed by endpoint titration. The titers were determined individually from each of the three parallel wells, and the bars represent the mean values ± SD. The numbers above the bars give the fold difference between the mock-treated and IFN-treated cells. Virus inoculation and following incubation were carried out at 15 °C for all the experiments.

The effect of IFN pretreatment on ISAV replication was further compared by studying the RNA levels of ISAV4 s6 in IFN-treated and untreated ISAV4-infected ASK cells using qPCR (Fig. 3B). Pretreatment with IFNa1, IFNb and IFNc reduced the ISAV4 s6 RNA levels 7.4-, 1.7- and 3.9-fold relative to untreated cells, respectively.

Western blot analyses revealed that all three IFNs also inhibited expression of ISAV4 HE and (Fig. 3C) NP (Fig. 3D) proteins. IFNa1 exhibited the highest inhibitory effect on NP and HE production followed by IFNc and IFNb, respectively. The protein samples were run in three parallel wells (not shown), and the net intensities of the NP and actin-bands were measured on a Kodak image station. The NP band intensities were normalized against the intensity of the respective actin bands, and the percent reduction in the NP bands by IFNa1, IFNb and IFNc relative to untreated control samples was determined. IFNa1 reduced the NP band intensity by 93%, IFNb by 67% and IFNc by 89% (Fig. 3E).

Finally, it was determined that IFNa1, IFNb and IFNc inhibited the viral yields of ISAV4 8-, 2- and 7-fold, respectively (Fig. 3F).

3.4. Induction of Mx and ISG15 protein by ISAV4 and ISAV7

The finding that ISAV7 induced Mx protein more strongly than ISAV4 (Fig. 2C and D) inspired us to examine the Mx and ISG15 protein-induction by the two ISAV strains. ASK cells were infected with ISAV4 and ISAV7, and samples were harvested 24 h p.i. A MOI of 5 was used to ensure that most cells were synchronously infected at an early time point after virus inoculation. As seen in Fig. 4A and B, ISAV7 induced higher protein levels of both Mx and ISG15 than ISAV4. Very similar levels of ISAV NP were expressed, indicating that comparable amounts of ISAV4 and ISAV7 had infected the cells. To obtain quantitative data, the net intensities of the Mx-, ISG15- and actin-bands from three parallel wells were measured, and the Mx/actin and ISG15/actin ratios were calculated. As displayed in Fig. 4C and D, ISAV7 induced a 2.7-fold higher level of Mx and a 2.9-fold higher level of ISG15 than ISAV4. Finally, the induction of Mx gene expression by ISAV4 and ISAV7 was compared by qPCR (Fig. 4E). At 72 h p.i., ISAV4 and ISAV7 upregulated the Mx transcript levels 180- and 270-fold,

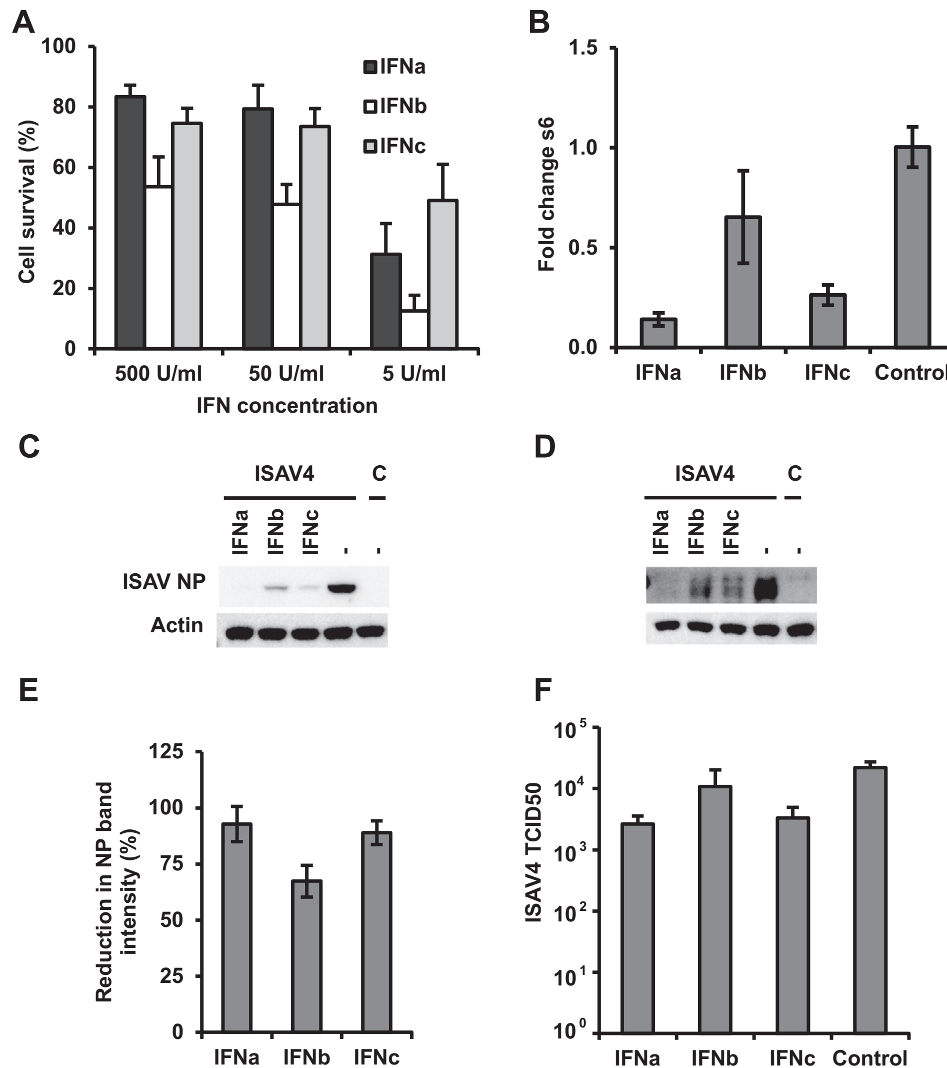

Fig. 3. Comparison of antiviral activity of IFNa1, IFNb and IFNc against ISAV4 in ASK cells. (A) CPE reduction assay. ASK were treated with various concentrations of IFN ($n = 4$) and infected with ISAV4 24 h later at a MOI of 1. When untreated control cells showed complete CPE, cell survival was estimated by crystal violet staining (optical density at 550 nm). (B) qPCR: ASK cells were treated with 1000 U/ml of IFNa1, IFNb and IFNc or left untreated ($n = 3$) and infected with ISAV4 (MOI = 1) 24 h later. RNA was sampled 72 h p.i. and the s6 RNA levels were determined by qPCR. (C) Western blot: ASK cells were treated with 1000 U/ml of the indicated IFNs, and infected with ISAV4 (MOI 1) 24 h later. The cells were harvested 72 h p.i., and the expression of NP and actin was analyzed. (D) Western blot analysis of ISAV HE was run on the samples presented in panel C. (E) The net intensities of the Western blot NP- and actin bands from three parallel wells were quantified, and the NP/actin ratio was calculated. The bars show the percent reduction in NP/actin ratio in the IFN-treated cells relative to untreated infected controls. (F) Supernatants from the qPCR experiment (panel B) were harvested at 72 h p.i., and the viral yields were determined by end-point titration in TO cells. The three replicate wells were titrated independently. In all the experiments virus inoculation and following incubation was performed at 20 °C. For panel A, B, E and F the mean values ± SD are given.

respectively, compared to untreated control cells. Taken together, these results suggests that ISAV7 induces the antiviral proteins Mx and ISG15 more potently than ISAV4 in ASK cells.

4. Discussion

The present work demonstrates that Atlantic salmon IFNa1 induces antiviral activity in ASK cells against both the high virulent strain ISAV4 and the low virulent strain ISAV7. However, at 15 °C, which is the optimal growth temperature of the virus, the antiviral effect was most pronounced at 24–72 h after infection, whereas the inhibitory effect seemed to decline four to five days after infection (Fig. 2A–D). This transient inhibition possibly explains why only a slight protection was observed in the CPE reduction assay at 15°, as it takes 8 days to reach complete ISAV-induced lysis of the cells. This may also be the reason why little antiviral activity of salmon IFN was observed in previous works, which only studied antiviral activity of IFN using the CPE reduction assay (Jensen and Robertsen, 2002; Kileng et al., 2007)

The transient antiviral effect of IFNa1 against ISAV could be a consequence of increasing levels of viral IFN antagonistic proteins as the viral replication progresses and/or degradation of IFN-induced proteins. The expression of viral proteins that limit the effects of antiviral proteins is well known from other orthomyxoviruses such as Influenza A and B. For example, the non-structural protein 1 (NS1) of influenza A is known to block the activation of PKR (Li et al., 2006a), whereas the NS1 protein of Influenza B has been demonstrated to inhibit the ISGylation process by interacting with ISG15 (Yuan and Krug, 2001). Among the ISAV proteins, s7ORF1 and s8ORF2 have been demonstrated to inhibit activation of the salmon IFNa1 promoter (García-Rosado et al., 2008), whereas s7ORF1 also inhibits the induction of the Mx promoter (McBeath et al., 2006). It is not known at present if

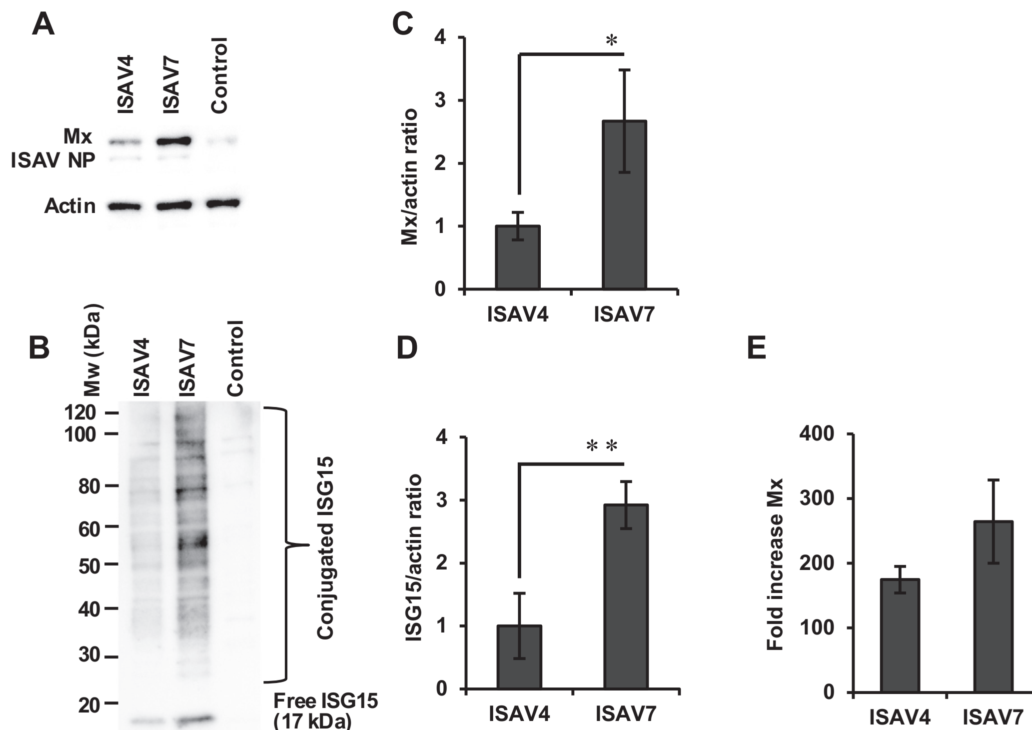

Fig. 4. Induction of Mx and ISG15 in ISAV4 and ISAV7 infected ASK cells. (A) ASK cells were infected with ISAV4 or ISAV7 (MOI 5) or mock-infected. The cells were harvested 24 h p.i. and the expression of Mx, actin and ISAV NP was analyzed by Western blotting. (B) The membrane from panel A was stripped, and the presence of ISG15 was analyzed. The net intensities of the Mx, ISG15 and actin bands from three parallel wells (not shown) were measured on a Carestream Molecular Imagine Software. The data show the Mx/actin ratio (C) and the ISG15/actin ratio (D) in ISAV7 and ISAV4-infected cells (set to 1). Mean values ± SD are given ($n = 3$). (E) mRNA levels of Mx were determined 72 h after ISAV4 and ISAV7-infection (MOI 1) in ASK cells by qPCR. Fold increase was calculated relative to uninfected cells. Mean values ± SD are given ($n = 3$). Student t test: $^{**}p < 0.01$; $^{*}p < 0.07$. In all the experiments the infection and incubation was carried out at 15 °C.

one or both of these proteins also inhibit the function of antiviral proteins, but this is an interesting subject for future studies. A high turnover rate of certain IFN-induced antiviral proteins might also explain the observed transient effect of IFNa1. This does not include Mx protein, which was strongly expressed at each time points in the virus-infected cells pretreated with IFNa1 (Fig. 2C and D). Mx proteins are, however, known to be particularly stable due to their ability to form large stabilizing aggregates (Haller et al., 2007). Other antiviral proteins are less stable, and the half-life of human PKR is for instance just 6.6 h (Liu et al., 2007).

In this work we also compared the antiviral activity of IFNa1, IFNb and IFNc against ISAV4. IFNd was not included, since this IFN subtype has previously been demonstrated to not confer antiviral activity against IPNV (Svingerud et al., 2012). IFNa1 and IFNc showed similar antiviral activity against ISAV4 in the CPE reduction assay and in the viral yield reduction assay, whereas IFNc displayed a slightly lower ability to reduce s6 RNA levels and HE and NP protein levels than IFNa. By contrast, IFNb displayed less antiviral activity than both IFNa1 and IFNc in all assays (Fig. 3). The higher antiviral effect of IFNa/IFNc compared to IFNb is in agreement with earlier work, where Atlantic salmon IFNa1 and IFNc displayed higher antiviral activity than IFNb against IPNV (Svingerud et al., 2012). In zebrafish, IFNa (IFNΦ1) and IFNc (IFNΦ2 and IFNΦ3) homologs signal *via* two different receptors (Aggad et al., 2009; Levraud et al., 2007). As suggested previously, the lower activity of IFNb may be due to lower expression of a putative IFNb receptor compared to putative IFNa and IFNc receptors, differences in the intracellular signaling pathways downstream of the receptor(s), or lower affinity for an IFN-receptor shared by IFNa, IFNb and IFNc (Svingerud et al., 2012). At present the nature and number of type I IFN receptors in Atlantic salmon are, however, unknown.

The replication of the high virulent ISAV4 and low virulent ISAV7 strains was inhibited to a similar degree by IFNa1 pretreatment at 24 and 72 h after virus inoculation. However, at 120 h p.i. the replication of ISAV7 was somewhat more strongly inhibited (2.4-fold *versus* 1.3-fold) than that of ISAV4 in the experiments conducted at 15 °C (Fig. 2A and B). By contrast, IFNa1 seemed to inhibit ISAV4 more strongly than ISAV7 when the virus inoculation and the subsequent cell incubation were performed at 20 °C (Fig. 1C and D). Whether these differences in IFN-sensitivity between the two viral strains play a role in the *in vivo* virulence of the two strains is uncertain.

Interestingly, ISAV7 induced about 3-fold higher protein levels of Mx and ISG15 than ISAV4 (Fig. 4A–D), and a 1.5-fold higher induction of Mx gene transcripts in ASK cells (Fig. 4E). An interesting subject for future studies is thus to examine if there is a difference in the IFN-pathway antagonizing properties of ISAV4 compared to ISAV7. The s7ORF1 proteins of ISAV4 and ISAV7 differ by four amino acids, whereas the s8ORF2 proteins differ by one amino acid, and these substitutions might affect the IFN antagonistic activities of the proteins (Markussen et al., 2008). In contrast to our study, Schiøtz et al. (2009) reported that ISAV4 induced Mx- and ISG15 gene expression more strongly (about 1.3-fold) than ISAV7 (Schiøtz et al., 2009). In both the present study and in the study by Schiøtz et al., the differences in gene expression between ISAV4 and ISAV7 were thus quite modest. The cause for the discrepancies could be that the ISAV4 and/or ISAV7 have evolved new traits during *in vitro* passaging in one or both of the studies. We did, however, confirm that the low and high virulent traits of ISAV7 and ISAV4 were still intact; in an *in vivo* challenge trial, 39 out of 40 Atlantic salmon presmolts were still alive 27 days after an intraperitoneally injection with ISAV7 (one thousand particles), while only twelve out of 40 fish survived an injection with the same amount of ISAV4

(results not shown). The discrepancies could further be caused by a difference in the amounts of ISAV4 *versus* ISAV7 used to infect the cells in one or both of the studies, which in turn would affect the virus-induced gene and protein expression. To control that comparable amounts of ISAV4 and ISAV7 had infected the ASK cells, we included a 4 h p.i. time point in our qPCR studies (Fig. 2A and B) to monitor the expression of viral s6 RNA early after infection. These data suggested that highly comparable levels of ISAV4 and ISAV7 had infected the cells. The virus control in the Western blot studies similarly revealed that highly comparable levels of ISAV NP was expressed in the ISAV4 and ISAV7-infected cells 24 h post infection (Fig. 4A). Interestingly, a recent study using two ISAV strains of European genotype showed that a low virulent ISAV strain upregulated Mx and other immune- and IFN-related genes to a higher degree than a high virulent strain (Workenhe et al., 2009).

In conclusion, the present work demonstrates that Atlantic salmon IFNa1, IFNb and IFNc exhibit a strong antiviral activity against ISAV in cell culture at early time points after infection. The effect of IFN is transient, which might explain earlier difficulties to firmly demonstrate inhibitory activity against ISAV. There was not a consistent difference between high- and low-virulent ISAV4 and ISAV7 strains in their sensitivity to the IFN-induced antiviral state *in vitro*, but ISAV4 appear to induce lower levels of Mx and ISG15 protein than ISAV7.

Conflict of interest

There are no actual or potential conflicts of interest.

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