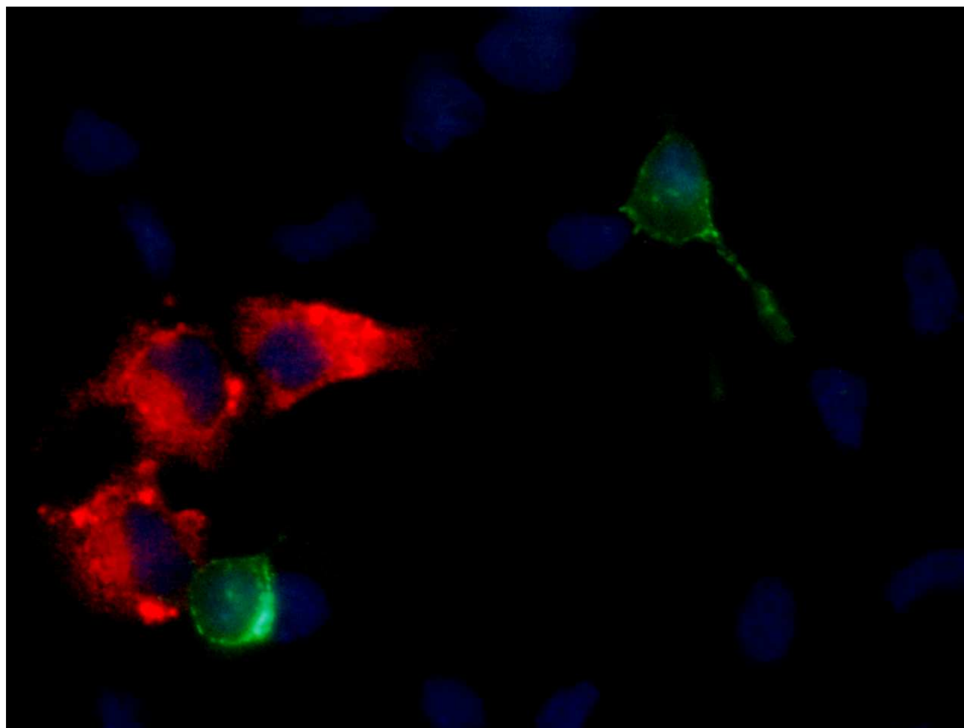
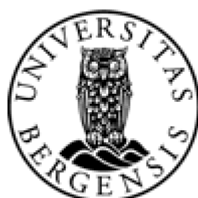
Co-infection with Norwegian Salmonid Alphavirus (NSAV) and Infectious Pancreatic Necrosis Virus (IPNV) in Chinook Salmon Embryo Cells (CHSE-214).



Thesis for the degree

Master of Science in Aquamedicine

Sven Amund Skotheim



Department of Biology

University of Bergen, Norway

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Abstract

Infectious pancreatic disease (IPN) and pancreas disease (PD) of salmon are viral diseases caused by Infectious Pancreatic Necrosis Virus (IPNV) (*Birnaviridae*) and Salmonid Alphavirus (SAV) (*Togaviridae*). Both IPNV- and SAV infections induce lesions in pancreas tissue/cells and are frequently detected from the same individual; hence it is possible that the viruses target the same cell types and therefore might interfere with each other during such infections. In the present study, Chinook Salmon Embryo Cells (CHSE-214) were experimentally co-infected with SAV and IPNV and infections were studied by IFAT, real-time RT- PCR and by viral end-point titration. Real-time RT-PCR was also used to examine to what extent the viruses up-regulated key transcripts (IFN and Mx) in the cellular antiviral immune response.

IFAT and end-point titration indicated that SAV to some extent inhibited IPNV replication , whereas IPNV did not affect SAV infections notably. Furthermore, the experiments demonstrated that key transcripts (IFN and Mx) in the cellular antiviral immune system were affected by the infections. Interestingly, transcription of these mRNAs were up-regulated in SAV infected, but not in IPNV infected cells, which could provide a possible explanation to the observed differences in the ability to interfere with the other virus.

Introduction

Pancreas Disease (PD) and Infectious Pancreas Necrosis (IPN)

Infectious Pancreatic Necrosis (IPN) is a disease of salmonids and a number of other fish species. An infectious aetiology for IPN was proposed (Wood, Snieszko, and Yasutake, 1955) and in 1960 infectious pancreatic necrosis virus (IPNV) was isolated in cell culture (Wolf et al., 1960). The agent is a virus in the family *Birnaviridae* (Dobos and Roberts, 1983), and was initially considered to cause diseases in rainbow trout (*Onchorhyncus mykiss)* and brook trout (*Salvelinus fontinalis*) fry. However, outbreaks may occur in Atlantic salmon during fresh and salt-water life stages. (Munro et al., 2006; Roberts and Pearson, 2005; Smail et al., 2006). Although IPN was believed to be a disease of salmonids, IPNV has been isolated from a variety of both freshwater and marine fish species, in addition to some cartilaginous vertebrates and invertebrates (Hill, 1982; Hill and K., 1995)

IPN diseases cause many histopathological changes in the infected salmonids. Thus, severe massive necrosis of acinar cells with nuclear pyknosis, karyorrhexis, and occasionally basophilic cytoplasmic inclusion are observed in pancreas (Smail et al., 1995). IPN affected rainbow trout and Atlantic salmon may also show congestion and necrosis of liver tissues (Taksdal, Stangeland, and Dannevig, 1997).

There is a wide variation in the degree of pathogenicity among different IPNV isolates. Mortality in rainbow trout fry vary between 0-90% due to infection with avirulent and highly virulent strains, respectively. (Sano et al., 1992).

Infectious pancreatic necrosis and Pancreas disease (PD) are two fish diseases with partly similar pathology. Pancreatic damage is common in both diseases (Munro et al., 1984; Smail et al., 1995). It is also known that these two viruses can act simultaneously on the same locality and in the same fish.

Pancreas Disease (PD) in Atlantic salmon (*Salmo salar*) has been known since 1976 (Munro et al., 1984). The disease was given its name due to histopathological changes in affected fish, which include acute necrosis in the exocrine pancreas (Munro et al., 1984), and cardiac- and skeletal myopathy (Ferguson, Rice, and Lynas, 1986). During the late 80s and early 90s, PD was diagnosed from Ireland and the west cost of USA (Kent and Elston, 1987; Murphy et al., 1992). The first report of PD in Norway was made in 1989 (Mcvicar, 1990; Poppe, Rimstad, and Hyllseth, 1989) and Murphy et al. (1992) proposed an infectious aetiology for pancreas disease, and in 1995, togavirus-like particles were successfully isolated from PD affected Atlantic salmon (Nelson et al., 1995). Morphologically the isolated virus resembled members of the *Togaviridae*, and the name Salmon Pancreas Disease Virus (SPDV) was proposed. Sequence analysis of cDNA clones from gradient-purified SPDV placed the virus among the *Alphavirus* genus, and it was the first *Alphavirus* described from fish (Weston et al., 1999).

A disease with similar histopathology to PD was described from freshwater reared rainbow trout (*Onchorhyncus mykiss*) in France, and given the name Sleeping Disease (SD) due to the unusual behavior of affected rainbow trout (Boucher P., 1994). The virus responsible for SD was isolated by (Castric et al., 1997), and the nucleotide also sequence classified the virus as an atypical *Alphavirus* of the *Togaviridae* (Villoing et al., 2000). Sequence analysis, monoclonal antibody reactivity and serological comparison of SPDV and SDV suggested that they were both distinct subtypes of a new *Alphavirus* species, for which the name salmonid Alphavirus (SAV) was proposed (Weston et al., 2002). Further studies have shown that virus isolated from PD in Norway differed from SPDV (SAV) and SDV (SAV2), this virus has been named Norwegian Salmonid Alphavirus (NSAV or SAV3) (Hodneland et al., 2005)

Traditionally, Salmonid alphavirus diseases have been distinguished into PD and SD, since disease outbreaks of these two viruses occur in saltwater and freshwater fish, respectively. The Norwegian outbreaks, reported as PD, have only occurred in the marine production phase of salmonids.

Salmonid Alphavirus structure and replication

The *Alphavirus* genus is well studied and characterized, but little work has been done with regard to molecular characterization of SAV *in vitro*. The genome size is similar to other alphaviruses at approximately 11900 nt. It consists of a positive sense single-stranded, non-segmented RNA that is divided into two open reading frames (ORF) with a 5`-terminal cap and a 3`-terminal poly (A) tract. The two-thirds of the genome at the 5`-terminal contains an ORF that codes for non-structural proteins (nsP), termed nsP1, nsP2, nsP3 and nsP4. The second ORF in the 3`-terminal (one-third of the genome) encodes a 26S sub-genomic mRNA that produces five structural proteins, termed capsid protein (C protein), E3, E2, 6K, and E1 (Hodneland et al., 2005; Villoing et al., 2000; Welsh et al., 2000; Weston et al., 1999). Recently, the existence of a ribosomal -1 frameshift site in the alphavirus structural polyprotein, that gives rise to the transframe protein TF has been shown (Firth et al., 2008).

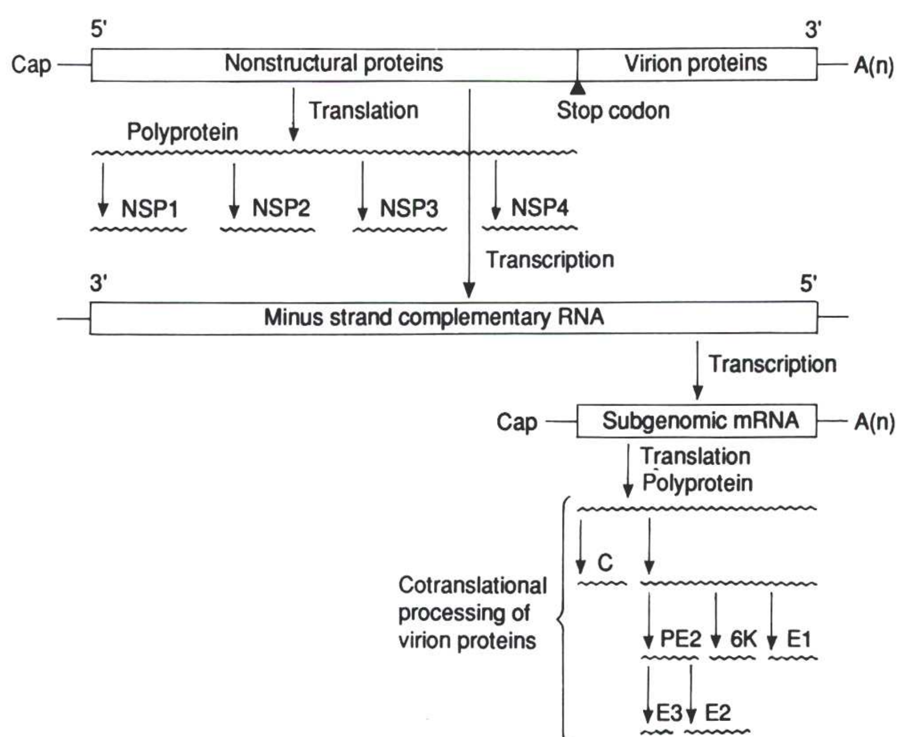

Fig. 1: Alphavirus replication. Figure from (White and Fenner, 1994).

Alphaviruses have a nucleocapsid consisting of 240 copies of the C protein, which is surrounded by a lipid envelope in which 240 copies each of two transmembrane glycoproteins (E1 and E2) are located. The E2 protein produces a stable heterodimer together with the E1 protein. Three E2-E1 heterodimers form one glycoprotein spike. The spikes are used for recognition, binding and entry into host cells (Strauss and Strauss, 1994). The low pH in the endosome forces the envelope proteins to undergo a structural change that makes the fusion between the viral envelope and the endosomal membrane possible (Strauss and Strauss, 1994). Fusion between the two membranes (i. e. cell infection) results in a release of the nucleocapsid into the cytoplasm (Baker, Olson, and Fuller, 1999). The uncoated viral genome serves as a messenger RNA for the synthesis of the nonstructural or replication proteins and as a template for the synthesis of the complementary 42S minus strand (Fig. 1). The 5` two thirds of the 42S alphavirus genome are translated into a single polyprotein P1234 that is autoproteolytically cleaved by nsP2; this cleaving results in a replicase complex consisting of P123 and nsP4. A RNA-dependent RNA polymerase complex, formed by these proteins, transcribes the genome into full-length 42S minus-strand RNA-templates. The proteinase level in the cell will increase after infection, making the RNA-dependent RNA polymerase complex unstable, and the complex will further be divided into nsP1, nsP2 and nsP3. This dividing gives a replicase complex that only produces (+) strand RNAs (Strauss and Strauss, 1994).

The last part of the genome, termed 26S sub-genomic mRNA, uses the full-length 42S minus strand for the protein synthesis. Translation of the structural polyprotein proceeds through the 26S mRNA that is used as template for production of a C-p62-6K-E1 polyprotein (Strauss and Strauss, 1994). After the capsid protein is autoproteolytically cleaved from this polyprotein it will associate with genomic 42S RNA in the cytoplasm, resulting in an icosahedral nucleocapsid structure (Garoff, Sjoberg, and Cheng, 2004; Strauss and Strauss, 1994). The remaining p62-6K-E1 contains a signal sequence that results in the translocation of the polypeptide to the membrane of the rough endoplasmic reticulum (RER) (Garoff et al., 1990; Garoff, Simons, and Dobberstein, 1978). In the endoplasmatic reticulum (ER) oligosaccharides covalently bind to the polypeptide, following a proteolytic cleavage into p62, 6K and E1 (Liljestrom and Garoff, 1991). An interaction between the P62, 6K and E1 in the ER gives heterodimeric complexes, before being transported to the Golgi complex. The glycoproteins will accumulate in the plasma membrane of host cell after the transport through the Golgi complex. P62 is oligomerized into E2 and E3 during transport through the Golgi

complex (Strauss and Strauss, 1994). It is suggested that the cytoplasmic nucleocapsid diffuses freely to the same site on the plasma membrane where the viral glycoproteins are embedded (Garoff, Sjoberg, and Cheng, 2004). At the plasma membrane there will be binding in a 1:1 molar ratio between the cytoplasmic C-terminus of the E2 in the glycoprotein spike and the newly arrived nucleocapsids. This will trigger the final assembly and budding of new viruses will occur (Garoff and Cheng, 2001).

Infectious Pancreas Necrosis Virus structure and replication

Infectious pancreatic necrosis virus (IPNV) belongs to the genus *Aquabirnavirus* in the family *Birnaviridae*. Electron microscopy studies have shown that the IPNV is about 55-65 nm in diameter (Dobos et al., 1979). The non-enveloped icosahedral capsid of IPNV contains two segments (A and B) of double-stranded RNA, which codes for 5 viral proteins (Havarstein et al., 1990). (Fig.2)

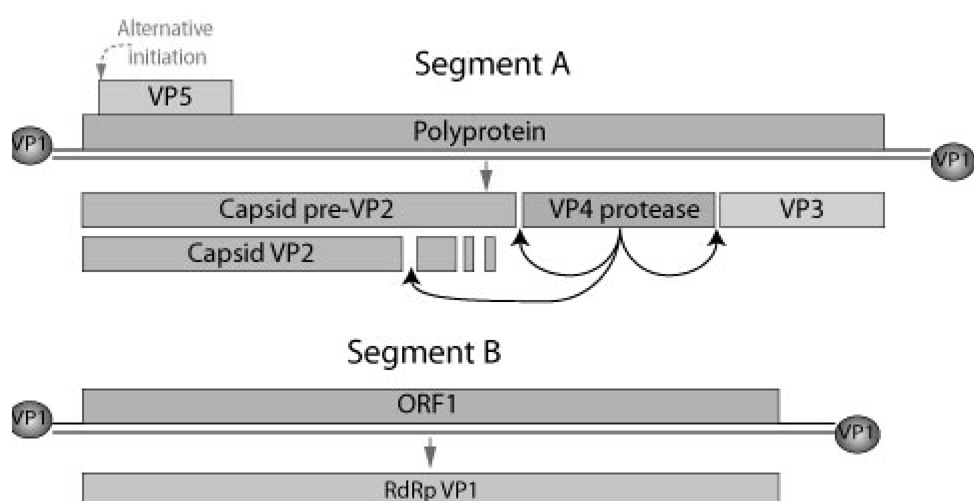

Fig. 2: genome organization of Infectious pancreatic necrosis virus (IPNV) (ViralZone. Swiss Bioinfomatics)

Genome segment A contains two overlapping open reading frames (ORFs), the shortest one located at the 5` end and encoding the non-structural protein VP5, (Duncan et al., 1987b; Havarstein et al., 1990) which has been detected in IPNV infected cells (Magyar and Dobos, 1994). It has been demonstrated that the VP5 protein is not essential for viral replication in cell culture (Weber et al., 2001), but Hong and Wu (2002) showed that VP5 was able to

induce anti-apoptotic functions in CHSE-214 cells. Apoptosis is the process of programmed cell death in response to a variety of stimuli, and is considered to be a part of the innate immune response to virus infection, limiting the time and cellular machinery available for viral replication (Everett and McFadden, 1999).

The additional larger ORF in segment A encodes a 106-kDa polyprotein that is processed into the mature VP2 and VP3 structural proteins through the proteolytic activity of VP4 (non-structural protein) (Duncan et al., 1987a; Petit et al., 2000). The determinants for virulence and cell culture adaption is associated with VP2 which is a major capsid protein (Song et al., 2005).

Genome segment B encodes a non-structural protein (VP1) that constitutes the RNA dependent RNA polymerase (RdRp) (Duncan et al., 1991). Two forms of RdRp are present in the virion, as free protein and covalently linked to the 5` ends of the two segments (VPg) (Calvert et al., 1991). It has been demonstrated *in vitro* that VP1 is guanylated and incorporated into a complex that serves as a primer for RNA synthesis (Xu, Si, and Dobos, 2004).

Two cell components, both specific and non-specific, are involved in the attachment of IPNV to the cellular membrane of host cells, (Kuznar et al., 1995). There is little or no information about the function of these two molecules. However, it has been proposed that VP2 is the cell attachment protein for the virion (Dobos, 1995a). It is suggested that the entry of IPNV, like many other naked viruses, is achieved by endocytosis. (Couve, Kiss, and Kuznar, 1992).

IPNV replication occurs in the cytoplasm of infected cells and synthesis of cellular proteins is not inhibited by this process (Saint-Jean, Borrego, and Perez-Prieto, 2003). Virus RNA in IPNV infected cells is transcribed by the virion RdRp, that is primed by VP1 and proceeds via an asymmetric, semiconservative strand-displacement mechanism (Dobos, 1995a; Dobos, 1995b). There is little or no information on minus strand RNA synthesis.

Major aspects associated with IPNV replication, such as particle assembly and interference with host cells are poorly understood. Villanueva et al., (2004) showed that two different sized viral particles were present during the IPNV infective cycle. Non-infectious, immature particles (66 nm) appeared immediately after RNA synthesis and viral dsRNA in infected

cells was detected simultaneously with this particle. This indicates that the viral assembly takes place right after the dsRNA replication has begun. Subsequently, the smaller (60 nm) and mature infectious virions are generated through proteolytic cleavage of the viral precursors within the capsid. (Villanueva et al., 2004). There is little available information about the viral assembly and release of IPNV, but in infected cells the assembly takes place in the cytoplasm and the release occurs via cell lysis (Hjalmarsson and Everitt, 1999).

Expression of interferon in response to virus infection

Interferons (IFNs) are proteins (cytokines) induced by virus entry into cells and secreted to play a major role in the defence against virus infections in vertebrates (Samuel, 2001). IFN-like activity was identified in fish as early as in 1965 and has since been detected in a variety of fish species including salmonids (Robertsen, 2006). On the basis of gene sequences, protein structure and functional properties two families of IFNs (type I and type II) can be distinguished (Kontsek, Karayianni-Vasconcelos, and Kontsekova, 2003; Pestka, Krause, and Walter, 2004; Samuel, 2001). IFN type I includes the classical IFN-α/β, which are induced by viruses in most cells, whereas IFN type II is identical to IFN-γ (Samuel, 2001). The information on IFN-α/β pathway proteins in teleost fish is limited, but the innate interferon system is similar to that in mammals (Robertsen, 2006).

Two IFN genes (IFN-α1 and IFN-α2) have been cloned from Atlantic salmon. Gene structure, promoter, antiviral activity and effect on expression of Mx and ISG15 (interferon stimulated gene) have also been characterized in these two genes (Bergan et al., 2006; Robertsen et al., 2003; Rokenes, Larsen, and Robertsen, 2007). IFN type I is considered to be one of the earliest non-specific anti-viral immune factors to be induced in fish (Robertsen, 2006).

For SAV it is known that type I IFN is induced *in vitro* in a number of cell types (Gahlawat, Ellis, and Collet, 2009), and *in vivo*, high levels of IFN-α/β are associated with systemic replication of a number of terrestrial alphaviruses (Klimstra et al., 1999; Ryman et al., 2000; Ryman et al., 2002; White et al., 2001).

An interferon induced antiviral effect against IPNV has been shown *in vitro* (Robertsen et al., 2003). The same authors have also shown *in vitro* a link between Mx protein expression and inhibition of IPNV (Jensen and Robertsen, 2002; Larsen, Rokenes, and Robertsen, 2004; Nygaard et al., 2000). An antiviral activity induced by Atlantic salmon Mx1 protein (ASMx1) has been shown to inhibit IPNV replication. CHSE-214 cells that expressed ASMx1 constitutively was found to inhibit virus induced cytopathic effect (CPE) and viral synthesis together with a reduction in the transcription of viral RNA (Larsen, Rokenes, and Robertsen, 2004).

Suppression of host responses by alphaviruses

Alphaviruses are capable of antagonizing the induction of stress and innate immune responses in cell cultures (Aguilar, Weaver, and Basler, 2007; Breakwell et al., 2007; Frolova et al., 2002). In a number of terrestrial alphaviruses the activities of nsP2 protein has this ability (Breakwell et al., 2007; Frolova et al., 2002). In other terrestrial alphaviruses the capsid proteins have been found to be responsible for this ability (Aguilar, Weaver, and Basler, 2007; Garmashova et al., 2007a). Interestingly, these proteins were also found to be the primary mediators of host cell transcription and translation shut off in cell cultures used for analysis of IFN induction. Virus mutants in these studies were selected for Nsp2 non-cytopatogenicity (Frolova et al., 2002) or no capsid protein expression and these mutants did not shut off host cell functions and induced IFN-α/β. (Aguilar, Weaver, and Basler, 2007; Garmashova et al., 2006; Garmashova et al., 2007b).

Suppression of host response by IPNV

Host cells treated with salmon rIFN-α1 after IPNV infection have shown that IPNV was able to inhibit IFN signalling (Collet et al., 2007; Jorgensen et al., 2007). Another study showed that the viral protein synthesis of IPNV was reduced if CHSE-214 cells were pre-treated with salmon IFN. This reduction prevents the process where pVP2 matures into VP2. The cells pre-treated with IFN were compared with cells infected with IPNV before IFN treatment; the results showed that IPNV was able to antagonize the antiviral state in cells infected with IPNV prior to IFN treatment (Skjesol A., 2009). The same authors showed that there is a

correlation between the time of IPNV infection and IFN treatment; the weakening of IFN signalling was more prominent the longer post-infection IFN was added. This indicates that IPNV is able to antagonize the cells innate immune responses. VP4 and VP5 reduced the IFN-induced expression more than other proteins tested, suggesting that these molecules are the strongest candidates for counteracting the IFN response (Skjesol A., 2009).

Aims of the study

SAV and IPNV frequently occur as co-infections in Norwegian Atlantic salmon, and it is likely that they may share common target cells (Munro et al., 1984; Smail et al., 1995). The aim of this study was to clarify how SAV- and IPNV replication might interfere with each other in Chinook Salmon Embryo Cells (CHSE-214) during *in vitro* co-infections. Another objective was to find out if the viruses were able to induce a non-specific antiviral response in the host cells, and to see if this might inhibit or block infection of the other virus. Such knowledge about innate immune responses and antiviral mechanisms could potentially be exploited in future vaccine strategies.

Material and Methods

Cell culture

Chinook Salmon Embryo (CHSE-214) cells were cultured in 175 cm^2 Nunclon™ bottles containing 30 ml Eagels minimum essential medium (EMEM) supplemented with 10 % foetal bovine serum (FBS), L-glutamine (4 mM), gentamicin (50 µg ml^{-1}), 1% non-essential amino acids and 1 % Hepes buffer concentration 2 pH at 20°C for 5-7 days until an approximately 80% monolayer was obtained. Infected cells were grown in 2% FBS maintenance medium (medium with 2% FBS instead of 10%) in 14°C. For virus propagation the CHSE-214 cell line was grown in 75 cm^2 Nunclon™ bottles infected with virus isolate (passage 22 for SAV and passage 20 for IPNV). The virus infected bottles were incubated for 7-8 days, until virus-induced cytopathic effect (CPE) was evident and the supernatant was collected and stored at -80 °C.

Virus isolates

The SAV3 isolate (SAVH 20/03) passage 22, used in this study was collected from infected salmon in Vik, Hordaland (Hodneland et al., 2005). The IPNV of the N1 strain, serotype SP was also used in this study (Christie et al., 1988). The N1 isolate has been shown to give low mortality in *in vivo* experiments. It is therefore classified as a low virulence strain (Sano et al., 1992).

Antibodies

Antibodies used in the present study included a monoclonal antibody (N1-B9) against the IPNV VP2 protein (Frost et al., 1995) and a polyclonal antibody against the E2 protein in SAV (E2-pTe200) (Ottem, 2005). Bound antibodies were detected using Alexa dye 488-labelled anti-rabbit IgG (Molecular probes) and/or Alexa dye 555-labelled anti-mouse IgG (Molecular probes).

The E2-pTE200 antibody against SAV was adsorbed on confluent CHSE-214 cells that were fixed 4% paraformaldehyde. Antibody diluted 1:10 in with phosphate buffered saline (PBS) containing 0.1 % natriumazid was incubated for 24 h at 4 °C in a 175 cm2 bottle of fixed CHSE-214 cells 4%. The diluted antibody was transferred into a new bottle of cells and incubated for 1 h. at room temperature. This last step was done twice. Finally the antibody was collected and stored at -20 °C until use.

Immunofluorescence antibody test (IFAT)

Indirect immunofluorescence staining was performed on IPNV and SAV infected CHSE-214 cells grown in 96 wells plates (Nunclon) and on coverslips in 24 well plates (Nunclon). Following incubation for 7-9 days, the cells were washed once in 1xPBS and fixed in 4 % paraformaldehyde for 20 min. Fixed cells were then permeabilized and dehydrated in a methanol-PBS series of 25, 50, 75 and 100 % methanol, before they were rehydrated using the same Methanol-PBS series in opposite direction. After blocking with 5 % skimmed milk powder in PBS for 30 min the cells were incubated for 1 h with the primary antibodies (SAV and IPNV) diluted in the blocking solution. Secondary antibodies were diluted in blocking solution; and diluted 1:1000. Alexa fluor 555 goat-anti mouse IgG was used for detection of antibodies bound with IPNV and Alexa Fluor 488 goat anti-rabbit used with the SAV antibody, PBS with 5 % FBS was used for washing between each step, three washes per step. During the last washing step a DAPI (4',6-diamidino-2-phenylindole) staining was added to stain nuclei. DAPI was diluted 1:6000 in PBS and added the cells for 2 minutes. By testing the different primary antibodies with different dilutions best dilution was determined.

Endpoint titration by IFAT

The viral titrations were performed on CHSE-214 cells grown in 96-well plates. The growth medium was removed and 150 µl maintenance medium was added to each well. The viral suspensions were diluted from 5^{-1} to 5^{-12} in maintenance medium (2% FBS) and with 50 µl of each dilution, which comprised a further dilution of 1:4. After 7-8 days incubation at 15 °C, the wells were subjected to IFAT. The 50% tissue culture infective dose (TCID50) was

estimated by the end-point titration method (Karber, 1931). The infective titers of both viruses involved in this study were equal.

IFAT detection of cells co-infected with SAV and IPNV

In this experiment Nunclon™ 24 well culture plates with CHSE-214 cells grown on coverslips were used. The coverslips with CHSE-214 cells were exposed for either SAV or IPNV, or combinations of these. The study consists of five different experimental groups (listed in table 1) performed in triplicate.

Table 1: Overview of experimental groups used in this study.
The group code stated in this table will be used to distinguish between groups throughout **this study.**

Groups	T_0	T_{24}	Group description
I	Mock	SAV	Single SAV infection
II	IPNV	SAV	IPNV infection, prior to co-infection with SAV 24 h. pi.
III	Mock	IPNV	Single IPNV infection
IV	SAV	IPNV	SAV infection, prior to co-infection with IPNV 24 h. pi.
V	Mock	SAV-IPNV	Simultaneously co-infection by SAV and IPNV
VI	Mock	Mock	Negative controls

Table 2: Overview of experimental groups used in this study (with half the virus concentration).

Groups	T_0	T_{24}	Group description
I (2)	Mock	SAV	Single SAV infection
II (2)	IPNV	SAV	IPNV infection, prior to co-infection with SAV 24 h. pi.
III (2)	Mock	IPNV	Single IPNV infection
IV (2)	SAV	IPNV	SAV infection, prior to co-infection with IPNV 24 h. pi.
V (2)	Mock	SAV-IPNV	Simultaneously co-infection by SAV and IPNV
VI (2)	Mock	Mock	Negative controls

All groups were fixed in 4% paraformaldehyde 48 h after the start of the experiment. IFAT and DAPI staining was then performed. There were three parallels for each group. The experiment was later repeated using half the virus concentrations (Table 2).

The coverslips from each group were removed from the wells and placed on a slide containing 5 µl SlowFade® Gold antifade reagent (Invitrogen) prior to microscopic examination. About 800-1000 cells were counted on each coverslip and the percentage of cell infected by the respective viruses in group I, II, III, IV, and V were estimated. This procedure was done with all of the coverslips in each of the three parallels. The percentages of infected cells are presented as the mean of the three parallels.

Experimental groups I, III (single infected) and VI were also used to investigate if SAV and IPNV induced apoptosis in CHSE-214 cells. In these three groups 100 cells were observed for each of the three parallels, and the average percentage of apoptotic cells was calculated. Cells with nuclei fragmentation visualized by DAPI staining were categorized as apoptotic cells. DAPI staining allows the visualization of cells whose nuclei integrity is lost. According to this it is able differentiate possible apoptotic and non-apoptotic CHSE-214 cells.

The IFAT study showed that there was less cell density in the wells infected with IPNV for 24 h, therefore, the cell densities in groups I, III and VI were compared. Four pictures were taken from each coverslip with a camera connected to the microscope, the number of cells counted and the cell number compared with each other.

Relative quantification by real-time RT-PCR and efficiency tests

A time series experiment where the cells were infected as described above was performed in 24 well culture plates. The cell layers in the wells were harvested for real-time RT-PCR analysis. In this experiment the cells were exposed for viruses/virus-combinations (table 1) for various time lengths prior to harvest/sampling at 6, 17, 41 and 110 h. For each time point there were three parallels with three negative controls. The supernatant from time 110 was collected and used in an endpoint titration, as described above. The cell layers in the wells were harvested 1 ml of TRI-reagent and stored at – 80 c, prior to RNA extraction and real-time RT-PCR analysis. Total RNA was extracted as described by (Devold et al., 2000).

The concentration of total RNA in each sample was measured in a NanoDrop ND-1000 spectrophometer. In order to have the same concentration of total RNA in the 1-step real-time RT-PCR reactions the differences of extracted RNA were adjusted by diluting samples with

the appropriate volume of double distilled nuclease free water. The adjusted RNA samples were spiked with a fixed amount of transcribed RNA from *Halobacterium salinarum* (SAL), which was used as an external control for the 1-step real-time RT-PCR. While the cellular elongation factor 1α (ElA) were used as internal control.

Verso^{TM} 1-step QRT-PCR ROX Kit was used for the real time RT-PCR assays. The reaction mixture for verso^{TM} 1-step QRT-PCR ROX Kit, was as follows RT-enhancer 0,625 µl, ROX master-mix 6,25 µl, enzyme mix 0,125 µl, primers 0,5 µl, probes 0.25 µl, 2 µl RNA template and DEPC-water added to a final volume of 12.5 µl. PCR was performed in an ABI 7500 sequence detection system (Applied Biosystems). The reaction was 15 min at 50 °C (Reverse Transcriptase step), 15 min at 95 °C (Polymerase activation step) followed by 45 cycles of 95 °C for 15 seconds (DNA-dissociation) and 60 °C for one minute (annealing and elongation). The PCR efficiency (E) for each essay (nsP1, IPN, Mx, IFN, SAL and ElA) was calculated from the slope of standard curves derived from five-fold RNA (triplicate) dilution series in yeast tRNA (Invitrogen) ($E=10^{-(1/slope)}$) (Pfaffl, 2001).

Mean normalized expression (MNE) values for SAV, IPNV (nsP1), Mx and IFN and standard errors were calculated using Q-gene which is based on Ct-values of triplicate runs and PCR-efficiency (Muller et al., 2002; Simon, 2003). The threshold was set to 0.02 for all assays when obtaining C_t-values. Negative template control and negative Trizol control were used for all assays in order to reduce the possibility of false positives. Primers and probes used towards the respective genes are listed in table 3. Two separate experiments were performed in order to test reproducibility.

Table 3: Primers and probes used in real-time RT-PCR analysis.

Primer and Probes	Target gene	Direction	Sequence (5`-3`)	Ref.
NSP1-F	**Non-structural p. 1**	**Fwd**	CCGGCCCTGAACCAGTT	(Hodneland and Endresen, 2006)
NSP1-R	**Non-structural p. 1**	**Rev**	GTAGCCAAGTGGGAGAAAGCT	
NSP1-Probe	**Non-structural p. 1**	**Probe**	TCGAAGTGGTGGCCAG	
IPNV-F		**Fwd**	ACCCCAGGGTCTCCAGTC	(Nylund, *Unpublished*)
IPNV-R		**Rev**	GGATGGGAGGTCGATCTCGTA	
IPNV-Probe		**Probe**	TCTTGGCCCCGTTCATT	
MX- F	Mx	**Fwd**	TGCAACCACAGAGGCTTTGAA	(Haugland et al., 2005)
MX- R	Mx	**Rev**	GGCTTGGTCAGGATGCCTAAT	
MX- probe	Mx	**Probe**	TGGCACAAGAGGTGGACCCTGAAGG	
INF- α- F	Interferon alpha	**Fwd**	TGGACGATTTCCTCAACATTCTAGA	(Fridell., *Unpublished*)
INF- α- R	Interferon alpha	**Rev**	TTCTTGAAGTAGCGTTTCAGTCTCTT	
INF- α- probe	Interferon alpha	**Probe**	CCTTAAATCCTGTGTATCACCTGCCATGAA	
EL1A-elaf	Elongation factor 1 α	**Fwd**	CCCCTCCAGGACGTTTACAAA	(Olsvik et al., 2005)
EL1A-elam1	Elongation factor 1 α	**Rev**	ATCGGTGGTATTGGAAC	
EL1A-elar	Elongation factor 1 α	**Probe**	CACACGGCCCACAGGTACA	
Sal- F	16S rRNA, H. salinarum	**Fwd**	GGGAAATCTGTCCGCTTAACG	(Andersen et al., Unpublished)
Sal- R	16S rRNA, H. salinarum	**Rev**	CCGGTCCCAAGCTGAACA	
Sal- Probe	16S rRNA, H. salinarum	**Probe**	AGGCGTCCAGCGGA	

Results

Immunofluorescence antibody test (IFAT)

A polyclonal antibody against the E2 protein in SAV and a monoclonal antibody against the VP2 protein in IPNV have been made. Both antibodies showed to be valuable diagnostic tools in IFAT. Uninfected cells showed some background with these antibodies, but specific staining was not obtained. Early experiments showed that the polyclonal antibody against SAV had major background staining even at different dilutions. However, after adsorption of CHSE-214 cells most of the unspecific binding was eliminated, reducing the background. The antibody against SAV detected proteins located at the outside of the plasma membrane and in the ER, which is to be expected since the E2 protein is located at these sites. The results from the dilution series showed that the adsorbed E2 antibody had little background and specific staining at a 1:400 dilution (Fig 3). The antibody against IPNV (B9-N1) was raised against virus protein 2 (VP2) and detected little background; therefore eliminating the need for adsorption. The antibody also detected proteins located at the membrane in the cytoplasm, which is expected since VP2 proteins are located at this site. For the VP2 antibody a 1:800 dilution was chosen based on background and strength of specific signal (Fig. 4).

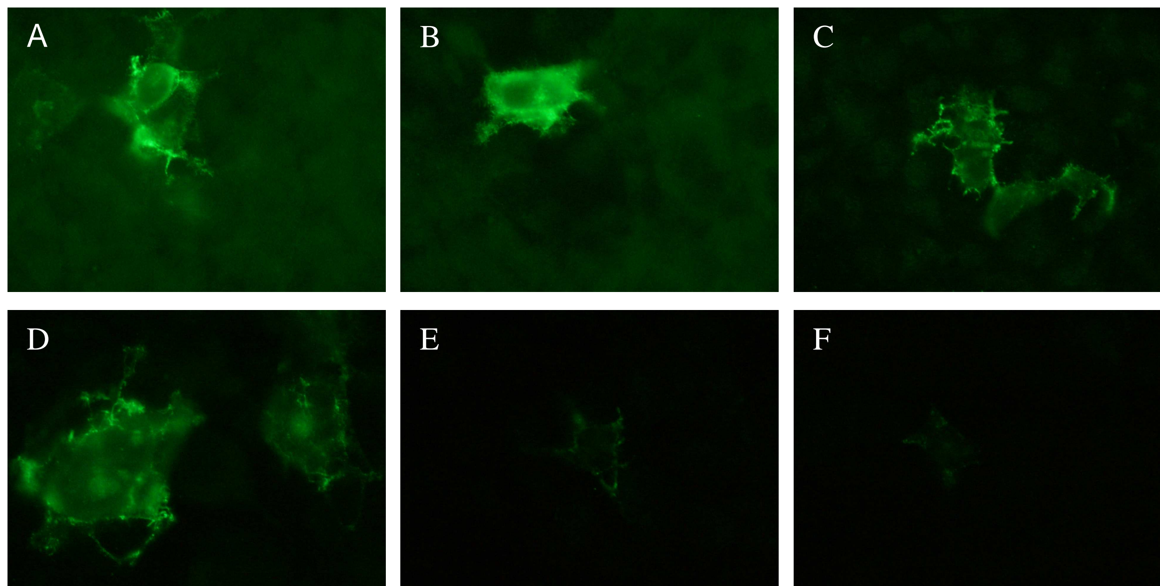

Fig. 3: Immunofluorescent staining of SAV infected CHSE-214 cells with different dilutions of a primary antibody raised against E2 protein (E2), (A) 1:50, (B) 1:100, (C) 1:200, (D) 1:400, (E) 1:800, (F) 1:1600. (All pictures are taken with 1/3 sec exposure and 2.80x gain)

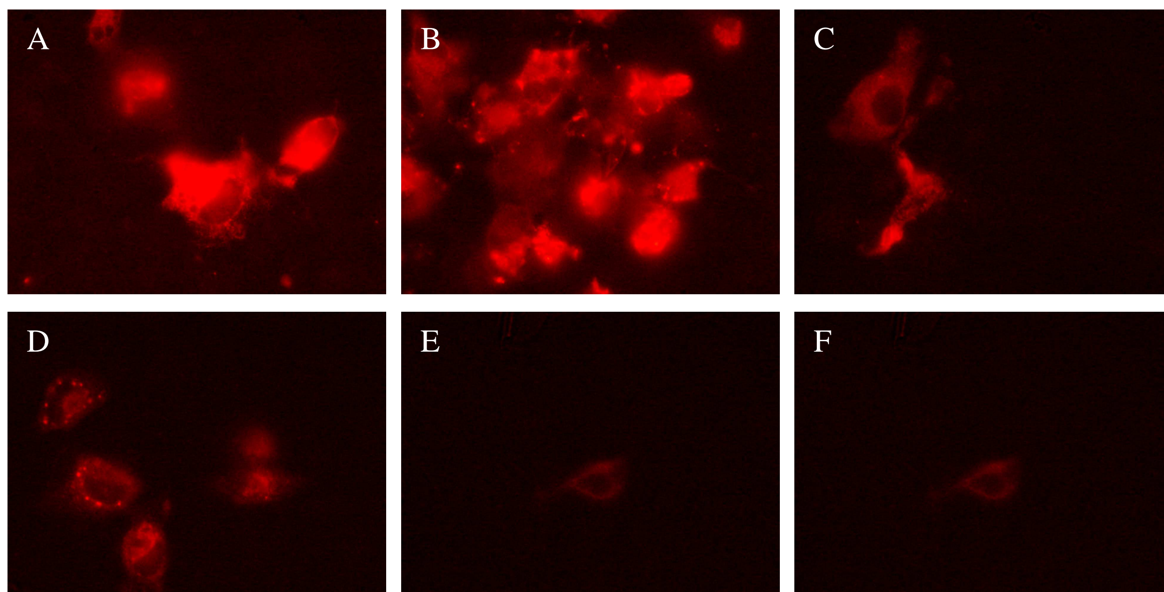

Fig. 4: Immunofluorescent staining of IPNV infected CHSE-214 cells with different dilutions of a primary antibody visualizing VP2/N1-B2, (A) 1:100, (B) 1:200, (C) 1:400, (D) 1:800, (E) 1:1600, (F) 1:3200 (all pictures are taken with 1/3 sec exposure and 2.80x gain)

IFAT experiments

IPNV Infected CHSE-214 cells developed a CPE, which is characterized by a progressive rounding of the cells. In order to establish the fraction of cells that developed CPE as a consequence of IPNV and SAV, the percentage of cells with nuclei fragmentation was found in group I, III and IV at 24 h. p. i. In groups I and III 75% cells showed fragmentation, while group IV (mock infected) had only 25% (Fig. 5).

To test the progress of infections, the cell densities for group I, III and IV were measured by cell counting. Group I and IV did not show any differences in cell densities. However, in group III there was about 30% less cell density compared to groups I and IV (Fig. 6).

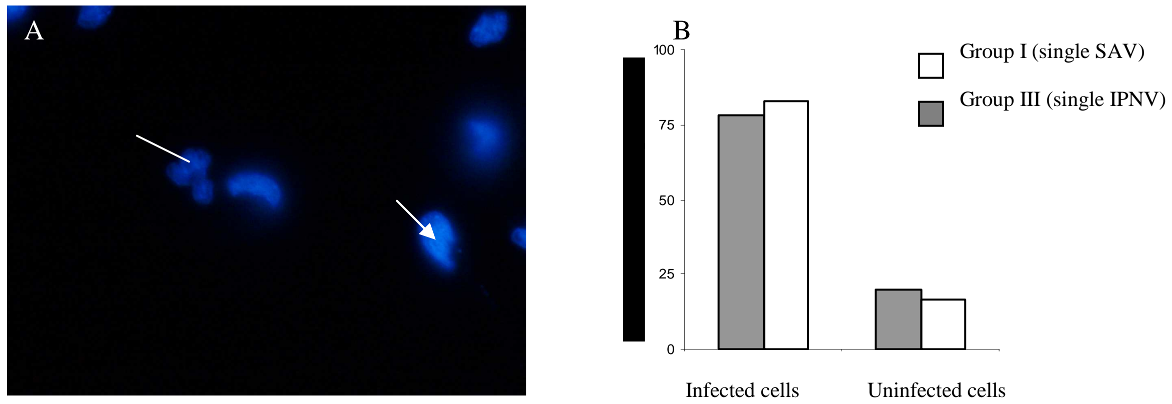

Fig. 5: Fragmented and non-fragmented nuclei in CHSE-214 cells.

A. (line) fragmented nuclei stained with DAPI, (arrow) normal nucleus stained with DAPI.

B. Percentage of fragmented and non-fragmented nucleus in SAV and IPNV infected cells compared to non-infected cells.

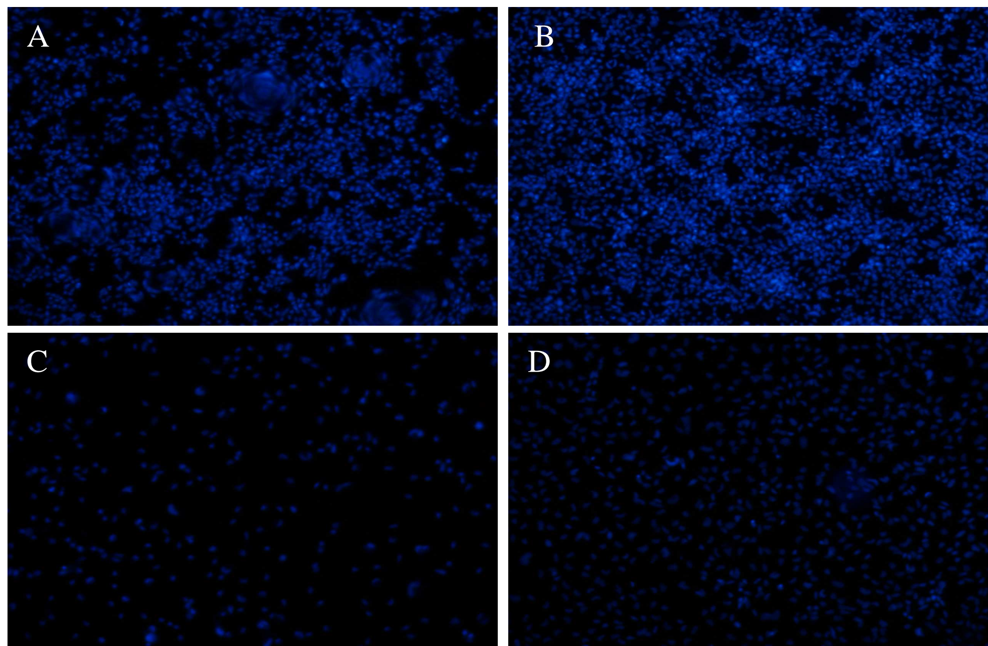

Fig. 6: DAPI stained CHSE-214 nuclei detected with IFAT. (picture A and B taken in the central part of the coverslip, C and D taken in the circumference of the coverslip.)

(A) and (C) CHSE-214 cells exposed for IPNV for 24 h. (B) and (D) uninfected CHSE-24 cells

Many viruses are able to infect the same cell, such infections are termed superinfection. The IFAT method was used to detect the superinfection of SAV and IPNV in the same cell. This was only observed in group V. Fig. 7 (A) shows two SAV infected cells in which the detected proteins are located at the membrane, (B) shows the same cells infected with IPNV. (C) and (D) shows the DAPI stained nucleus and all pictures merged together, respectively.

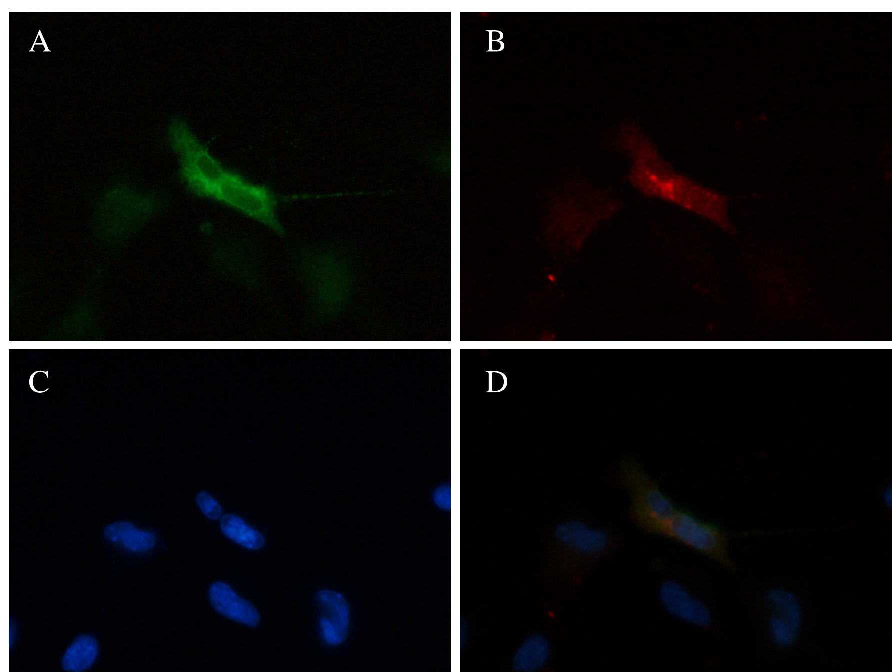

Fig. 7: CHSE-214 cells co-infected with SAV and IPNV (group V) visualized using IFAT and DAPI

A. SAV visualized with the E2 antibody

B. IPNV visualized with the VP2/N1-B2 antibody.

C. DAPI stained nucleus.

D. All pictures merged.

In the IFAT detection of cells infected with virus/virus combinations (Table 1), the percentage of infected cells after 24 h was estimated for each parallel. Together (all three parallels), there were about 2500 cells counted for group I, II, III, IV and V (Table 1). No infected cells were detected in group VI (uninfected control). Fig. 8 shows the mean percentage of the three parallels for SAV and IPNV infected cells. The range, which represents the difference in the parallel with lowest percentage and the parallel with highest percentage are given for both SAV and IPNV infected cells. The percentage of SAV infected cells in group I, II and V were compared. The results showed 32% infected cells in group I, 38% in group II and 45% in V.

The same procedure was done to find the percentage of IPNV infected cells in groups III, IV and V. Interestingly, there were no IPNV infected cells detected in group IV and V (groups challenged with SAV), and group III had only 6% IPNV infected cells.

In order to check the validity of these results the virus concentrations were dropped to the half for the virus/virus combinations (Table 2). When half the virus concentration was used the percentage of SAV infected cells in group I(2), II(2) and V(2) were reduced to 10%, 7% and 7%, respectively (fig. 8). Surprisingly, this reduction resulted in a higher percentage of IPNV infected cells, 9% IPNV infected cells in group III(2), and 3% in the two other groups (Fig. 8).

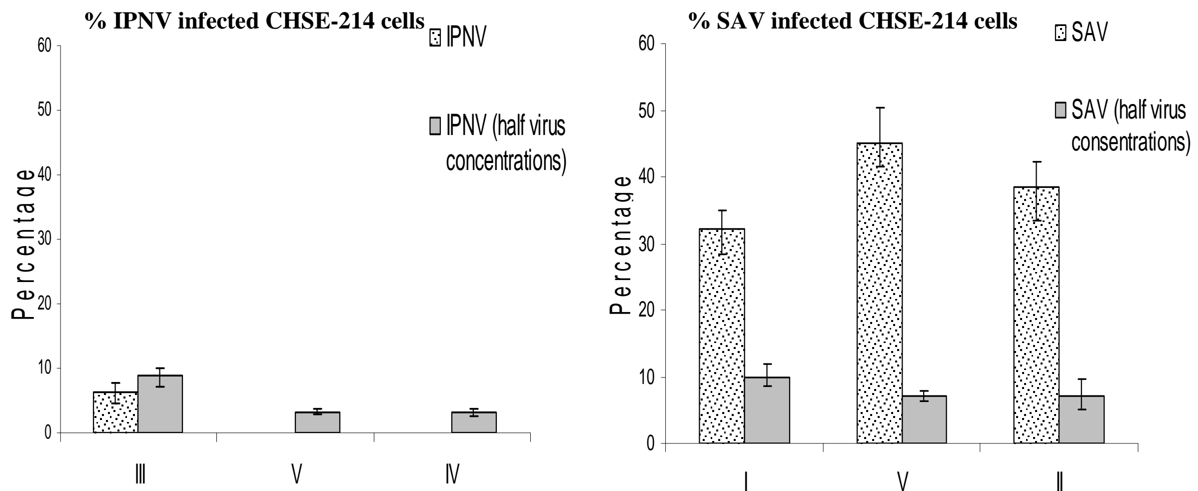

Fig. 8: Percentage of SAV and IPNV infected CHSE-214 cells in different virus combinations fixed after 24 h. The range from the three parallels is shown for each group.

Real-time RT-PCR analysis.

Standards curves were derived from five-fold dilution series for all assays in order to estimate PCR efficiencies. PCR effciency were calculated from slopes of standard curves (fig. 9). Slope of standard curve for elongation factor 1(ElA) are described is (Andersen et al., 2007).

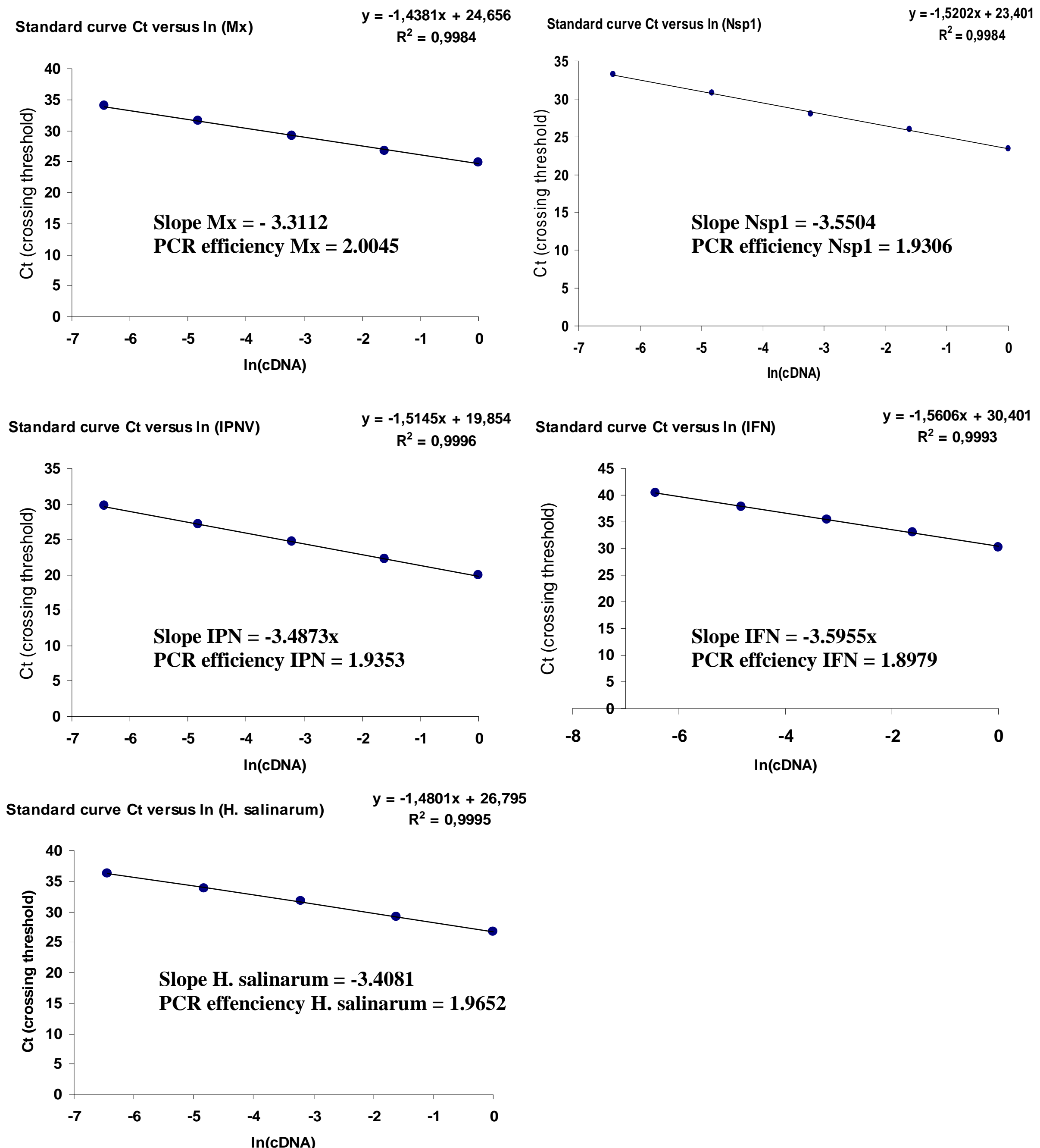

Fig. 9: Standard curves for the five different assays used in this study; standard curves for all assays are five fold diluted in yeast tRNA and run in triplicates.

Real-time RT-PCR analysis was performed on RNA extracted from two of the three parallels from the time series experiment and negative controls. The target genes used were Nsp1 normalized against SAL and ELA reference genes. Group I, II and V were compared in both duplicate 1 and 2. The MNE values for Nsp1 against both reference genes showed small differences in the three experimental groups in the time series.

MNE values in duplicate number 2 for both reference genes could be as much as 10 fold lower compared to duplicate number 1. The differences in group II observed in duplicate 1 were not reproducible in duplicate 2. Both, duplicate 1 and 2 independently of reference gene, showed that the nsP1 expression were at the highest after 17 h (Fig. 10).

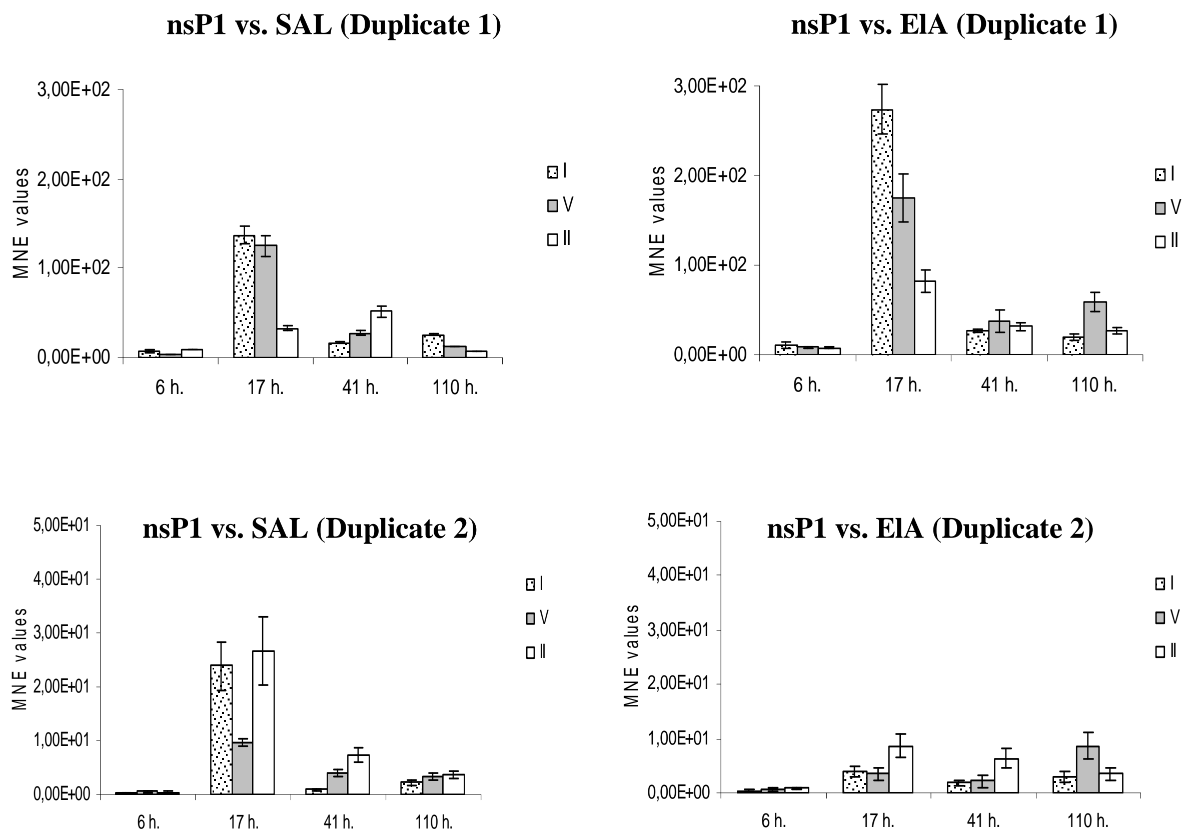

Fig. 10: Duplicate of MNE values for Nsp1 in CHSE-214 cells in the three different groups harvested at different time points; (I) SAV single infection, (V) simultaneously SAV/IPNV co-infection and (II) SAV infection after 24 h exposure to IPNV. The nsP1 was normalized against SAL and ELA reference genes. (The scale used for Duplicate 2 is adjusted in order to see the trend. However, it should be mentioned that duplicate 2 had much lower expression of nsP1).

Similarity, MNE values for IPNV showed that duplicate 1 and 2 did not have any measureable differences in the three groups. In duplicate 1 the MNE values for IPNV against both reference genes showed that RNA expression was highest at 17 h. In duplicate 2 this peak was only observed with SAL as reference gene (Fig. 11).

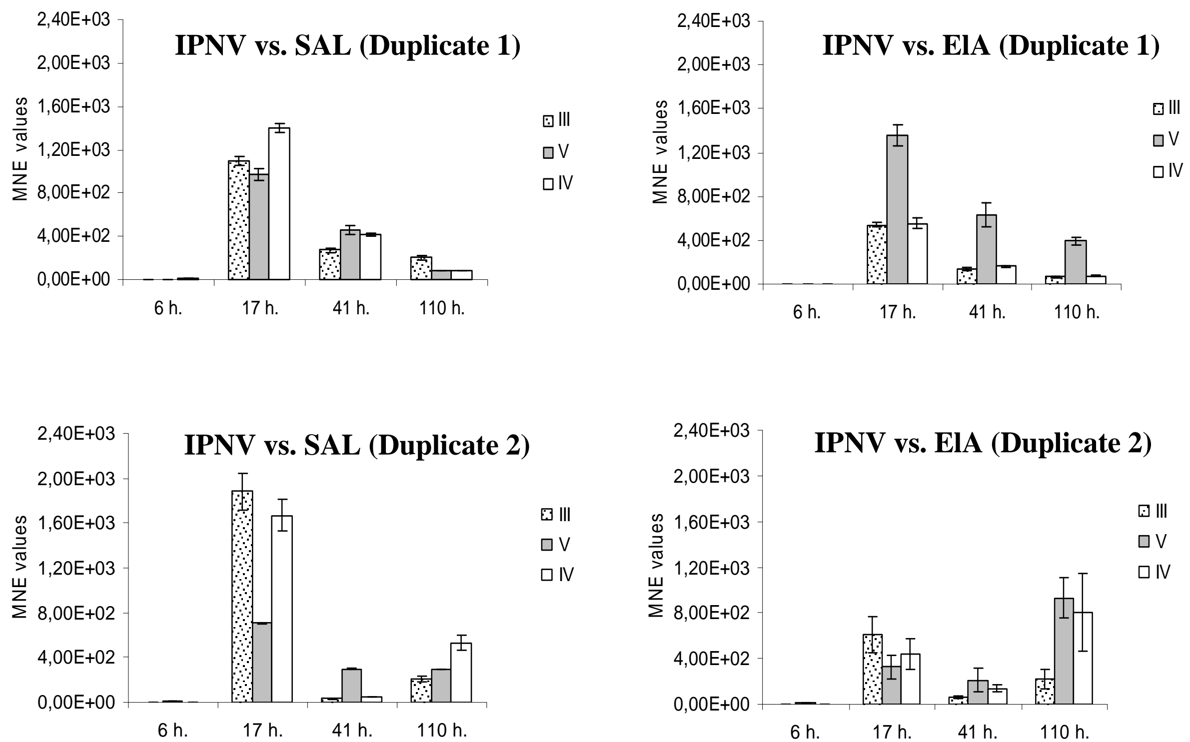

Fig. 11: Duplicate of MNE values for IPNV in CHSE-214 cells in group III, V and IV; (III) IPNV single infection, (V) simultaneously IPNV/SAV co-infection and (IV) IPNV infection after 24 h exposure to SAV. The IPNV was normalized against SAL and ELA reference genes.

Real-time RT-PCR analysis for IFN-α and Mx was performed with the same RNA as used in duplicate 1 (fig. 12). All six experimental groups were analyzed. Mean normalized expression (MNE) values for IFN showed that group I (single SAV) after 24 h induced a strong up-regulation of IFN-α, independently of reference genes. IPNV did not show to induce any measurable IFN up-regulation.

MNE values for Mx showed that group IV gave the highest Mx induction at 6 h, and that group I and V gave the highest induction at 17 h. Group III (single IPNV) did not induce Mx production at all. All these results were observed with both reference genes.

in group II at 17 h, however, the IFN-α up-regulation was much lower compared to group I, although this two groups induced almost the same amount of Mx.

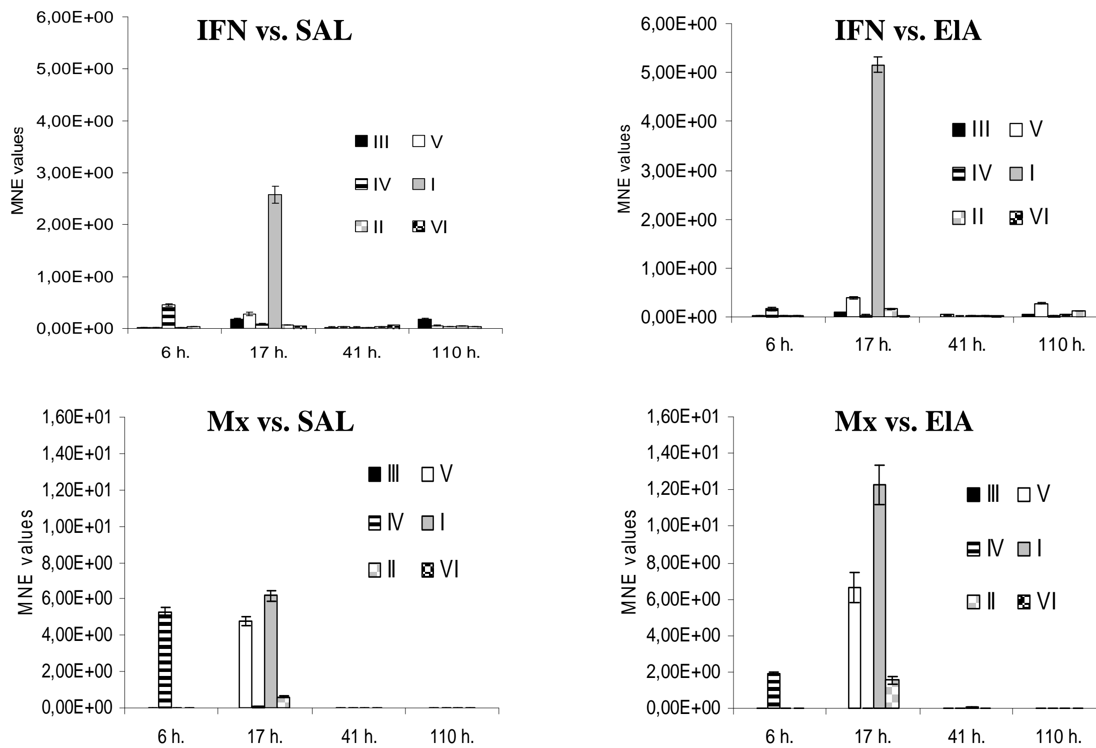

Fig. 12: MNE values in all six groups, with IFN-α and Mx as target genes towards two reference genes (H. salinarum and elongation factor 1).

End-point titration (110 hour, time series experiment)

Supernatant from all groups (Table 1) were collected from the time series experiment at 110 h., and endpoint titration was performed for both SAV and IPNV in all groups (Table 2).

In this study there were four parallels for each of the six groups, the viral titer were sat to be the last wells were over 50% of the wells had virus infected cells detectable with IFAT . Interestingly, the result showed that all SAV infected groups had the same amount of virus per ml supernatant. However, there were measurable differences in the groups infected with IPNV. As Table 1 shows, group III (single IPNV) had the highest amount of IPNV infectious particles. In group II, IV and V the amount of IPNV were 5, 5 and 25 times less compared to group III.).

Table 2: Virus per ml for SAV and IPNV in the six different groups, the supernatants used for titration were collected 110 h post the initial infection time.

	I	**II**	**III**	**IV**	**V**	**IV**
SAV	1,25E+06	1,25E+06	0	1,25E+06	1,25E+06	0
IPNV	0	2,50E+05	1,25E+06	2,50E+05	5,00E+04	0

Disscussion

Co-infections with birnaviruses and other viruses have been described earlier (Alonso, Rodriguez, and Perez-Prieto, 1999; Alonso, Rodriguez Saint-Jean, and Perez-Prieto, 2003; Johansen and Sommer, 2001; Saint-Jean and Perez-Prieto, 2007), but the possible interactions between birnaviruses and alphaviruses have not been examined. It is likely that these two viruses might affect the same individual fish, and target cells that are located in the pancreas tissue (Munro et al., 1984; Smail et al., 1995). The present work provides evidence that during such co-infections SAV interfere with IPNV replication in CHSE-214 cells, while IPNV does not notably affect SAV. It is shown that SAV induce INF-α and Mx in CHSE-214, while IPNV does not. This is in agreement with previous studies (A. Bratland, *Unpublished;* Jensen and Robertsen, 2002; Rokenes, Larsen, and Robertsen, 2007). The IFN-system may play a key role in the observed interference between these viruses.

Several diagnostic tests like the indirect immunofluorescence assay (IFAT), enzyme-linked immunosorbent assays (ELISA) and molecular diagnostic tests like real-time-RT-PCR have been developed for fish pathogens (Adams et al., 1995; Overturf, LaPatra, and Powell, 2001). These methods can be used to examine IPNV and SAV and also RNA or protein products. In the present study CHSE-214 cells experimentally co-infected with SAV and IPNV were studied by (I) IFAT, which detect specific proteins, (II) real-time RT- PCR which detect genomic segments of the virus, and, (III) viral end-point titration, which detect infectious virus particles. The IFAT method proved to be particularly useful since it allowed visual identification of infected cells. This made it possible to investigate if the viruses were able to infect the same cell. Although not commonly observed, these studies demonstrated that single cells where both the SAV E2 protein and the IPNV VP2 protein were expressed existed in the cell layer. This confirms that both SAV and IPNV replication, at least to the extent of expression of these proteins, may occur in the same cell. As part of the IFAT method, staining of cell nuclei with DAPI was performed. This provided information about the integrity of cell nuclei during infection, and suggested that infections with both SAV and IPNV lead to fragmentation of the nucleus. The result agrees with *in vitro* studies of IPNV infection which reported that morphological changes like fragmentation of nuclei could be seen in apoptotic cells as early as 6 h. p. i. of CHSE-214 cells (Hong et al., 1999). Apoptosis is morphologically characterized by cell shrinkage and hyperchromatic nuclear fragments (Wyllie, Kerr, and

Currie, 1980). Like for IPNV, it has been shown that terrestrial alphaviruses trigger apoptotic death of the infected host cell (Glasgow et al., 1997; Mastrangelo et al., 2000; Nava et al., 1998). More than one method should, however, be applied to confirm that SAV induce apoptosis in CHSE-214 cells.

By combining IFAT with real-time-RT-PCR for quantification of RNA, it was possible to obtain data about the percentage of infected cells as well as levels of chosen viral and host RNA transcripts. While IFAT showed that the percentage of IPNV infected cells in the cell layer were reduced during co-infections, a corresponding reduction in viral RNA levels could not be detected. By performing the real-time-RT-PCR experiments in duplicate runs, it could be demonstrated, however, that regulation in RNA levels was only in the 0-5 interval. Such small variations are difficult to measure due to uncertainties that are provided by the method. To measure expression kinetics of the selected viral and host RNA during infection, target transcripts were normalized against reference transcripts. This opens for discussion about the stability and reliability of the chosen reference genes. Ideal housekeeping gene should show a constant expression between individuals and different developmental stages, and it should be unaffected by any experimental treatment (Zhang, Ding, and Sandford, 2005). The internal housekeeping gene Elongation factor 1A (ElA) used, has previously been described for use as a reference gene in fish. E1A transcript is steadily expressed under a variety of terms, though its performance during a virus infection is not that well known and it is most likely dependant on virus species (Olsvik et al., 2005). Bratland, (*unpublished*) suggested ElA to be an acceptable reference gene towards nsP1. From previous studies, one can see that the expression of a number of the frequently used house keeping genes can vary under experimental circumstances (Radonic et al., 2004; Schmittgen and Zakrajsek, 2000; Vandesompele et al., 2002). A second reference gene (external) was therefore used. This consisted of an *in vitro* transcribed 900 nt RNA homologous to 16S rRNA of the bacterium *H. salinarum*, which was added to the samples after RNA extraction, thereby, not affected by pre-extraction handling or gene expression variations in the samples. Although the real-time-RT-PCR method was unable to detect reliable differences in viral RNA levels between the test groups, the final outcome of the infections found with endpoint titration showed that less infectious IPNV units were produced during co-infections than during single infections, while the production of infectious SAV units was not affected by presence of IPNV.

In addition to monitoring levels of viral RNA, the real-time-RT-PCR experiments demonstrated that key transcripts in the anti viral immune system (IFN and Mx) were affected by the infections. Interestingly, transcription of these mRNAs were up-regulated in SAV infected, but not in IPNV infected cells, providing a possible explanation to the observed differences in the ability to interfere with the other virus. Up-regulation of IFN and Mx during SAV infection is in agreement with previous studies (Bratland, *unpublished;* Gahlawat, Ellis, and Collet, 2009). Although the SAV genome is a single stranded RNA, all RNA viruses need to synthesize antisense RNA at some time during their replication cycle. This leads to potential presence of viral dsRNA, which is known to trigger many of the cellular responses to virus infection, including IFN-inducible enzymes (Jacobs and Langland, 1996). However, cells infected with IPNV, which has a double stranded RNA genome, induced no IFN-like activity. A possible explanation for this has been suggested by Skjesol (2009) who showed that VP4 and VP5 reduced the IFN-induced expression suggesting that these molecules are the strongest candidates for counteracting the IFN system. This concur with earlier studies that has demonstrated that IPNV induced neither IFN nor Mx during infection of TO cells, or CHSE-214 cells (Jensen and Robertsen, 2002; Rokenes, Larsen, and Robertsen, 2007). IPNV is, however, able to induce an IFN response both in other cell types, like rainbow trout gonad cell line and in vivo (Collet et al., 2007; de Sena and Rio, 1975; Jensen and Robertsen, 2000; McBeath et al., 2007; Saint-Jean and Perez-Prieto, 2007). The virus is also sensitive to presence of IFN-induced effector proteins. (Larsen, Rokenes, and Robertsen, 2004) transfected CHSE-214 cells with the Atlantic salmon Mx1 protein (ASMx1) and demonstrated an antiviral effect of these cells towards IPNV. Other studies have demonstrated that an anti-IPNV activity induced by IFN and Mx is obtained in a number of cell types (Jensen and Robertsen, 2002; Nygaard et al., 2000; Robertsen et al., 2003).

Effective vaccines against fish viruses have traditionally been difficult to develop (Biering et al., 2005). The majority of the commercial vaccines that are available at the moment contain inactivated disease agents. In vaccines against viral agents, the use of inactivated agents may be overly simplistic since the innate immune system, an important line of defence against viruses, in not activated. Effective response to and control of virus infection seems to require several levels of interactions between the innate and adaptive immune systems (Kang and Compans, 2009). Live attenuated vaccines provide a complete viral lifecycle and, thus, may overcome this problem through stimulation of responses that resemble those seen after natural

virus infection. Such vaccines could potentially have advantages over traditional vaccines also in aquaculture (Benmansour and de Kinkelin, 1997). A major disadvantage off attenuated vaccine, however, is the possibility that they will revert to a virulent form (Benmansour and de Kinkelin, 1997). A recombinant full-length SAV2 was recently published (Moriette et al., 2006). This study demonstrated that a recombinant strain of SAV2 (SD) that possessed little or no virulence in fish was able to protect rainbow trout against wild type SAV2. Moreover, it was demonstrated that SAV can replicate and express foreign RNAs, opening the possibility of using live SAV as a delivery vector of any antigen in fish.

The trigging of antiviral mechanisms (IFN and Mx) by SAV demonstrated in this study might be useful features in the development of recombinant alphavirus-based vaccines. In such a vaccine there will be a specific protection obtained by the antigens and a non-specific protection obtained by the reported up regulation of antiviral mechanisms in cells induced by wild type SAV. As this study shows this might be a future application against IPN and other diseases caused by viruses.

Conclusion and Future perspectives

This study showed that IFN-mediated antiviral activity and Mx expression could be induced by SAV, and that such induction would correlate with the inhibition of IPNV in the co-infections. IPNV did not up-regulate IFN and Mx, nor did it interfere with SAV, suggesting that the IFN-system may play a key role in the observed interference *in vitro* between these viruses. However, despite the reported correlation between IFN and Mx induction and inhibition of IPNV replication, it should be mentioned that other cytokines with antiviral activity also could be involved. The results of the present study were based entirely on *in vitro* experiments that can never fully reproduce *in vivo* conditions. Therefore, it would be of great interest to conduct co-infection experiments on salmonid fish in test the in vivo interference of these two viruses.

Adams, A., Thompson, K. D., Morris, D., Farias, C., and Chen, S. C. (1995). Development and Use of Monoclonal-Antibody Probes for Immunohistochemistry, Elisa and Ifat to Detect Bacterial and Parasitic Fish Pathogens. *Fish & Shellfish Immunology* **5**(8)**,** 537-547.

Aguilar, P. V., Weaver, S. C., and Basler, C. F. (2007). Capsid protein of eastern equine encephalitis virus inhibits host cell gene expression. *J Virol* **81**(8)**,** 3866-76.

Alonso, M., Rodriguez, S., and Perez-Prieto, S. I. (1999). Viral coinfection in salmonids: infectious pancreatic necrosis virus interferes with infectious hematopoietic necrosis virus. *Arch Virol* **144**(4)**,** 657-73.

Alonso, M., Rodriguez Saint-Jean, S., and Perez-Prieto, S. I. (2003). Virulence of infectious hematopoietic necrosis virus and Infectious pancreatic necrosis virus coinfection in rainbow trout (Oncorhynchus mykiss) and nucleotide sequence analysis of the IHNV glycoprotein gene. *Arch Virol* **148**(8)**,** 1507-21.

Andersen, L., Bratland, A., Hodneland, K., and Nylund, A. (2007). Tissue tropism of salmonid alphaviruses (subtypes SAV1 and SAV3) in experimentally challenged Atlantic salmon (Salmo salar L.). *Archives of Virology* **152**(10)**,** 1871-1883.

Baker, T. S., Olson, N. H., and Fuller, S. D. (1999). Adding the third dimension to virus life cycles: three-dimensional reconstruction of icosahedral viruses from cryo-electron micrographs. *Microbiol Mol Biol Rev* **63**(4)**,** 862-922, table of contents.

Benmansour, A., and de Kinkelin, P. (1997). Live fish vaccines: history and perspectives. *Developments in Biological Standardization 90***,** pp. 279–289.

Bergan, V., Steinsvik, S., Xu, H., Kileng, O., and Robertsen, B. (2006). Promoters of type I interferon genes from Atlantic salmon contain two main regulatory regions. *FEBS J* **273**(17)**,** 3893-906.

Biering, E., Villoing, S., Sommerset, I., and Christie, K. E. (2005). Update on viral vaccines for fish. *Dev Biol (Basel)* **121,** 97-113.

Boucher P., B. L. F. (1994). Sleeping disease of salmonids. *Bulletin of European Association of Fish Pathologists* **14,** 179-180.

Breakwell, L., Dosenovic, P., Karlsson Hedestam, G. B., D'Amato, M., Liljestrom, P., Fazakerley, J., and McInerney, G. M. (2007). Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. *J Virol* **81**(16)**,** 8677-84.

Calvert, J. G., Nagy, E., Soler, M., and Dobos, P. (1991). Characterization of the VPg-dsRNA linkage of infectious pancreatic necrosis virus. *J Gen Virol* **72 (Pt 10),** 2563-7.

Castric, J., Laurencin, F. B., Bremont, M., Jeffroy, J., Ven, A. L., and Bearzotti, M. (1997). Isolation of the virus responsible for Sleeping Disease in experimantally infected Rainbow trout (*Oncorhynchus mykiss*). *Bull. Eur. Ass. Fish. Pathol.* **17,** 27-30.

Christie, K. E., Havarstein, L. S., Djupvik, H. O., Ness, S., and Endresen, C. (1988). Characterization of a new serotype of infectious pancreatic necrosis virus isolated from Atlantic salmon. *Arch Virol* **103**(3-4)**,** 167-77.

Collet, B., Munro, E. S., Gahlawat, S., Acosta, F., Garcia, J., Roemelt, C., Zou, J., Secombes, C. J., and Ellis, A. E. (2007). Infectious pancreatic necrosis virus suppresses type I interferon signalling in rainbow trout gonad cell line but not in Atlantic salmon macrophages. *Fish Shellfish Immunol* **22**(1-2)**,** 44-56.

Couve, E., Kiss, J., and Kuznar, J. (1992). Infectious pancreatic necrosis virus internalization and endocytic organelles in CHSE-214 cells. *Cell Biol Int Rep* **16**(9)**,** 899-906.

de Sena, J., and Rio, G. J. (1975). Partial purification and characterization of RTG-2 fish cell interferon. *Infect Immun* **11**(4)**,** 815-22.

Devold, M., Krossoy, B., Aspehaug, V., and Nylund, A. (2000). Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout Salmo trutta after experimental infection. *Dis Aquat Organ* **40**(1)**,** 9-18.

Dobos, P. (1995a). The molecular biology of infectious pancreatic necrosis virus (IPNV) *In* "Annual Review of Fish Diseases", Vol. 5, pp. 25-54.

Dobos, P. (1995b). Protein-primed RNA synthesis in vitro by the virion-associated RNA polymerase of infectious pancreatic necrosis virus. *Virology* **208**(1)**,** 19-25.

Dobos, P., Hill, B. J., Hallett, R., Kells, D. T. C., Becht, H., and Teninges, D. (1979). Biophysical and Biochemical Characterization of 5 Animal Viruses with Bisegmented Double-Stranded-Rna Genomes. *Journal of Virology* **32**(2)**,** 593-605.

Dobos, P., and Roberts, T. E. (1983). The Molecular-Biology of Infectious Pancreatic Necrosis Virus - a Review. *Canadian Journal of Microbiology* **29**(4)**,** 377-384.

Duncan, R., Mason, C. L., Nagy, E., Leong, J. A., and Dobos, P. (1991). Sequence analysis of infectious pancreatic necrosis virus genome segment B and its encoded VP1 protein: a putative RNA-dependent RNA polymerase lacking the Gly-Asp-Asp motif. *Virology* **181**(2)**,** 541-52.

Duncan, R., Nagy, E., Krell, P. J., and Dobos, P. (1987a). Synthesis of the Infectious Pancreatic Necrosis Virus Polyprotein, Detection of a Virus-Encoded Protease, and Fine-Structure Mapping of Genome Segment-a Coding Regions. *Journal of Virology* **61**(12)**,** 3655-3664.

Duncan, R., Nagy, E., Krell, P. J., and Dobos, P. (1987b). Synthesis of the infectious pancreatic necrosis virus polyprotein, detection of a virus-encoded protease, and fine structure mapping of genome segment A coding regions. *J Virol* **61**(12)**,** 3655-64.

Everett, H., and McFadden, G. (1999). Apoptosis: an innate immune response to virus infection. *Trends in Microbiology* **7**(4)**,** 160-165.

Ferguson, H. W., Rice, D. A., and Lynas, J. K. (1986). Clinical pathology of myodegeneration (pancreas disease) in Atlantic salmon (Salmo salar). *Vet Rec* **119**(12)**,** 297-9.

Firth, A. E., Chung, B. Y., Fleeton, M. N., and Atkins, J. F. (2008). Discovery of frameshifting in Alphavirus 6K resolves a 20-year enigma. *Virol J* **5,** 108.

Frolova, E. I., Fayzulin, R. Z., Cook, S. H., Griffin, D. E., Rice, C. M., and Frolov, I. (2002). Roles of nonstructural protein nsP2 and Alpha/Beta interferons in determining the outcome of Sindbis virus infection. *J Virol* **76**(22)**,** 11254-64.

Frost, P., Havarstein, L. S., Lygren, B., Stahl, S., Endresen, C., and Christie, K. E. (1995). Mapping of Neutralization Epitopes on Infectious Pancreatic Necrosis Viruses. *Journal of General Virology* **76,** 1165-1172.

Gahlawat, S. K., Ellis, A. E., and Collet, B. (2009). Expression of interferon and interferon--induced genes in Atlantic salmon Salmo salar cell lines SHK-1 and TO following infection with Salmon AlphaVirus SAV. *Fish Shellfish Immunol* **26**(4)**,** 672-5.

Garmashova, N., Atasheva, S., Kang, W., Weaver, S. C., Frolova, E., and Frolov, I. (2007a). Analysis of Venezuelan equine encephalitis virus capsid protein function in the inhibition of cellular transcription. *J Virol* **81**(24)**,** 13552-65.

Garmashova, N., Gorchakov, R., Frolova, E., and Frolov, I. (2006). Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. *J Virol* **80**(12)**,** 5686-96.

Garmashova, N., Gorchakov, R., Volkova, E., Paessler, S., Frolova, E., and Frolov, I. (2007b). The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *J Virol* **81**(5)**,** 2472-84.

Garoff, H., and Cheng, R. H. (2001). The missing link between envelope formation and fusion in alphaviruses. *Trends Microbiol* **9**(9)**,** 408-10.

Garoff, H., Huylebroeck, D., Robinson, A., Tillman, U., and Liljestrom, P. (1990). The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. *J Cell Biol* **111**(3)**,** 867-76.

Garoff, H., Simons, K., and Dobberstein, B. (1978). Assembly of the Semliki Forest virus membrane glycoproteins in the membrane of the endoplasmic reticulum in vitro. *J Mol Biol* **124**(4)**,** 587-600.

Garoff, H., Sjoberg, M., and Cheng, R. H. (2004). Budding of alphaviruses. *Virus Res* **106**(2)**,** 103-16.

Glasgow, G. M., McGee, M. M., Sheahan, B. J., and Atkins, G. J. (1997). Death mechanisms in cultured cells infected by Semliki Forest virus. *Journal of General Virology* **78,** 1559-1563.

Haugland, O., Torgersen, J., Syed, M., and Evensen, O. (2005). Expression profiles of inflammatory and immune-related genes in Atlantic salmon (Salmo salar L.) at early time post vaccination. *Vaccine* **23**(48-49)**,** 5488-99.

Havarstein, L. S., Kalland, K. H., Christie, K. E., and Endresen, C. (1990). Sequence of the large double-stranded RNA segment of the N1 strain of infectious pancreatic necrosis virus: a comparison with other Birnaviridae. *J Gen Virol* **71 (Pt 2),** 299-308.

Hill, B. J. (1982). Infectious pancreatic necrosis virus and its virulence. *Microbial Diseases of Fish (ed. by R. J. Roberts)***,** pp. 91–114.

Hill, B. J., and K., W. (1995). Serological classification of infectious pancreatic necrosis (IPN) virus and other aquatic birnaviruses. *Annual Review of Fish Diseases***,** 5, 55–77.

Hjalmarsson, A., and Everitt, E. (1999). Identification of IPNV-specified components released from productively infected RTG-2 cells following massive cytopathic effect. *Archives of Virology* **144**(8)**,** 1487-1501.

Hodneland, K., Bratland, A., Christie, K. E., Endresen, C., and Nylund, A. (2005). New subtype of salmonid alphavirus (SAV), Togaviridae, from Atlantic salmon Salmo salar and rainbow trout Oncorhynchus mykiss in Norway (vol 66, pg 113, 2005). *Diseases of Aquatic Organisms* **67**(1-2)**,** 181-181.

Hodneland, K., and Endresen, C. (2006). Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan (R)). *Journal of Virological Methods* **131**(2)**,** 184-192.

Hong, J. R., Lin, T. L., Yang, J. Y., Hsu, Y. L., and Wu, J. L. (1999). Dynamics of nontypical apoptotic morphological changes visualized by green fluorescent protein in living cells with infectious pancreatic necrosis virus infection. *Journal of Virology* **73**(6)**,** 5056-5063.

Jacobs, B. L., and Langland, J. O. (1996). When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. *Virology* **219**(2)**,** 339-49.

Jensen, I., and Robertsen, B. (2002). Effect of double-stranded RNA and interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anemia virus and infectious pancreatic necrosis virus. *Fish Shellfish Immunol* **13**(3)**,** 221-41.

Jensen, V., and Robertsen, B. (2000). Cloning of an Mx cDNA from Atlantic halibut (Hippoglossus hippoglossus) and characterization of Mx mRNA expression in response to double-stranded RNA or infectious pancreatic necrosis virus. *J Interferon Cytokine Res* **20**(8)**,** 701-10.

Johansen, L. H., and Sommer, A. I. (2001). Infectious pancreatic necrosis virus infection in Atlantic salmon Salmo salar post-smolts affects the outcome of secondary infections with infectious salmon anaemia virus or Vibrio salmonicida. *Dis Aquat Organ* **47**(2)**,** 109-17.

Jorgensen, J. B., Johansen, A., Hegseth, M. N., Zou, J., Robertsen, B., Collet, B., and Secombes, C. J. (2007). A recombinant CHSE-214 cell line expressing an Mx1 promoter-reporter system responds to both interferon type I and type II from salmonids and represents a versatile tool to study the IFN-system in teleost fish. *Fish & Shellfish Immunology* **23**(6)**,** 1294-1303.

Kang, S. M., and Compans, R. W. (2009). Host responses from innate to adaptive immunity after vaccination: molecular and cellular events. *Mol Cells* **27**(1)**,** 5-14.

Karber, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch. Exp. Path. Pharmak* **162,** 480-3.

Kent, M. L., and Elston, R. A. (1987). Pancreas disease in net pen reard Atlantic salmon in North America. *Bull. Eur. Ass. Fish. Pathol.* **7,** 29-31.

Klimstra, W. B., Ryman, K. D., Bernard, K. A., Nguyen, K. B., Biron, C. A., and Johnston, R. E. (1999). Infection of neonatal mice with sindbis virus results in a systemic inflammatory response syndrome. *J Virol* **73**(12)**,** 10387-98.

Kontsek, P., Karayianni-Vasconcelos, G., and Kontsekova, E. (2003). The human interferon system: characterization and classification after discovery of novel members. *Acta Virol* **47**(4)**,** 201-15.

Kuznar, J., Soler, M., Farias, G., and Espinoza, J. C. (1995). Attachment and entry of infectious pancreatic necrosis virus (IPNV) into CHSE-214 cells. *Arch Virol* **140**(10)**,** 1833-40.

Larsen, R., Rokenes, T. P., and Robertsen, B. (2004). Inhibition of infectious pancreatic necrosis virus replication by atlantic salmon Mx1 protein. *J Virol* **78**(15)**,** 7938-44.

Liljestrom, P., and Garoff, H. (1991). Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. *J Virol* **65**(1)**,** 147-54.

Magyar, G., and Dobos, P. (1994). Evidence for the detection of the infectious pancreatic necrosis virus polyprotein and the 17-kDa polypeptide in infected cells and of the NS protease in purified virus. *Virology* **204**(2)**,** 580-9.

Mastrangelo, A. J., Hardwick, J. M., Bex, F., and Betenbaugh, M. J. (2000). Part I. Bcl-2 and Bcl-x(L) limit apoptosis upon infection with alphavirus vectors. *Biotechnology and Bioengineering* **67**(5)**,** 544-554.

McBeath, A. J., Snow, M., Secombes, C. J., Ellis, A. E., and Collet, B. (2007). Expression kinetics of interferon and interferon-induced genes in Atlantic salmon (Salmo salar) following infection with infectious pancreatic necrosis virus and infectious salmon anaemia virus. *Fish Shellfish Immunol* **22**(3)**,** 230-41.

Mcvicar, A. H. (1990). Infection as a primary cause of pancreas disease in farmed Atlantic salmon. *Bull. Eur. Ass. Fish. Pathol.***,** 10, 84-87

Moriette, C., Leberre, M., Lamoureux, A., Lai, T. L., and Bremont, M. (2006). Recovery of a recombinant salmonid alphavirus fully attenuated and protective for rainbow trout. *J Virol* **80**(8)**,** 4088-98.

Muller, P. Y., Janovjak, H., Miserez, A. R., and Dobbie, Z. (2002). Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* **32**(6)**,** 1372-4, 1376, 1378-9.

Munro, A. L. S., Ellis, A. E., Mcvicar, A. H., Mclay, H. A., and Needham, E. A. (1984). An Exocrine Pancreas Disease of Farmed Atlantic Salmon in Scotland. *Helgolander Meeresuntersuchungen* **37**(1-4)**,** 571-586.

Munro, E. S., Gahlawat, S. K., Acosta, F., and Ellis, A. E. (2006). In infectious pancreatic necrosis virus carrier Atlantic salmon, Salmo salar L., post-smolts, almost all kidney macrophages ex vivo contain a low level of non-replicating virus. *J Fish Dis* **29**(1)**,** 43-8.

Murphy, T. M., Rodger, H. D., Drinan, E. M., Gannon, F., Kruse, P., and Korting, W. (1992). The Sequential Pathology of Pancreas Disease in Atlantic Salmon Farms in Ireland. *Journal of Fish Diseases* **15**(5)**,** 401-408.

Nava, V. E., Rosen, A., Veliuona, M. A., Clem, R. J., Levine, B., and Hardwick, J. M. (1998). Sindbis virus induces apoptosis through a caspase-dependent, CrmA-sensitive pathway. *Journal of Virology* **72**(1)**,** 452-459.

Nelson, R. T., Mcloughlin, M. F., Rowley, H. M., Platten, M. A., and Mccormick, J. I. (1995). Isolation of a Toga-Like Virus from Farmed Atlantic Salmon Salmo-Salar with Pancreas Disease. *Diseases of Aquatic Organisms* **22**(1)**,** 25-32.

Nygaard, R., Husgard, S., Sommer, A. I., Leong, J. A., and Robertsen, B. (2000). Induction of Mx protein by interferon and double-stranded RNA in salmonid cells. *Fish Shellfish Immunol* **10**(5)**,** 435-50.

Olsvik, P. A., Lie, K. K., Jordal, A. E., Nilsen, T. O., and Hordvik, I. (2005). Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Mol Biol* **6,** 21.

Ottem, K. F. (2005). Expression of Norwegian Salmonid Alphavirus (NSAV) structural protein E2 in *E. coli*. *Master thesis in Aquamedicine. Department of Biology. Univeristy of Bergen.*

Overturf, K., LaPatra, S., and Powell, M. (2001). Real-time PCR for the detection and quantitative analysis of IHNV in salmonids. *Journal of Fish Diseases* **24**(6)**,** 325-333.

Pestka, S., Krause, C. D., and Walter, M. R. (2004). Interferons, interferon-like cytokines, and their receptors. *Immunol Rev* **202,** 8-32.

Petit, S., Lejal, N., Huet, J. C., and Delmas, B. (2000). Active residues and viral substrate cleavage sites of the protease of the birnavirus infectious pancreatic necrosis virus. *J Virol* **74**(5)**,** 2057-66.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29:e45**.

Poppe, T., Rimstad, E., and Hyllseth, B. (1989). Pancreas disease in Atlantic salmon (Salmo salar) post-smolts infected with infectious pancreatic necrosis (IPNV). *Bull. Eur. Ass. Fish. Pathol.***,** 9:83-85

Radonic, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W., and Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun* **313**(4)**,** 856-62.

Roberts, R. J., and Pearson, M. D. (2005). Infectious pancreatic necrosis in Atlantic salmon, Salmo salar L. *J Fish Dis* **28**(7)**,** 383-90.

Robertsen, B. (2006). The interferon system of teleost fish. *Fish Shellfish Immunol* **20**(2)**,** 172-91.

Robertsen, B., Bergan, V., Rokenes, T., Larsen, R., and Albuquerque, A. (2003). Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. *J Interferon Cytokine Res* **23**(10)**,** 601-12.

Rokenes, T. P., Larsen, R., and Robertsen, B. (2007). Atlantic salmon ISG15: Expression and conjugation to cellular proteins in response to interferon, double-stranded RNA and virus infections. *Mol Immunol* **44**(5)**,** 950-9.

Ryman, K. D., Klimstra, W. B., Nguyen, K. B., Biron, C. A., and Johnston, R. E. (2000). Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. *J Virol* **74**(7)**,** 3366-78.

Ryman, K. D., White, L. J., Johnston, R. E., and Klimstra, W. B. (2002). Effects of PKR/RNase L-dependent and alternative antiviral pathways on alphavirus replication and pathogenesis. *Viral Immunol* **15**(1)**,** 53-76.

Saint-Jean, S. R., Borrego, J. J., and Perez-Prieto, S. I. (2003). Infectious pancreatic necrosis virus: Biology, pathogenesis, and diagnostic methods. *Advances in Virus Research, Vol 62* **62,** 113-165.

Saint-Jean, S. R., and Perez-Prieto, S. I. (2007). Effects of salmonid fish viruses on Mx gene expression and resistance to single or dual viral infections. *Fish Shellfish Immunol* **23**(2)**,** 390-400.

Samuel, C. E. (2001). Antiviral actions of interferons. *Clin Microbiol Rev* **14**(4)**,** 778-809, table of contents.

Sano, M., Okamoto, N., Fukuda, H., Saneyoshi, M., and Sano, T. (1992). Virulence of Infectious Pancreatic Necrosis Virus Is Associated with the Larger Rna Segment (Rna Segment-a). *Journal of Fish Diseases* **15**(4)**,** 283-293.

Schmittgen, T. D., and Zakrajsek, B. A. (2000). Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J Biochem Biophys Methods* **46**(1-2)**,** 69-81.

Simon, P. (2003). Q-Gene: processing quantitative real-time RT-PCR data. *Bioinformatics* **19**(11)**,** 1439-40.

Skjesol A., A. T., Hegseth M., Robertsen B., Jørgensen J. (2009). The interplay between infectious pancreatic necrosis virus (IPNV) and the IFN system: IFN signaling is inhibited by IPNV infection. *Virus Res.*

Smail, D. A., Bain, N., Bruno, D. W., King, J. A., Thompson, F., Pendrey, D. J., Morrice, S., and Cunningham, C. O. (2006). Infectious pancreatic necrosis virus in Atlantic salmon, Salmo salar L., post-smolts in the Shetland Isles, Scotland: virus

identification, histopathology, immunohistochemistry and genetic comparison with Scottish mainland isolates. *J Fish Dis* **29**(1)**,** 31-41.

Smail, D. A., McFarlane, L., Bruno, D. W., and McVicar, A. H. (1995). The pathology of an IPN-Sp sub-type (Sh) in farmed Atlantic salmon, Salmo salar L, post-smolts in the Shetland Isles, Scotland. *Journal of Fish Diseases* **18**(6)**,** 631-638.

Song, H., Santi, N., Evensen, O., and Vakharia, V. N. (2005). Molecular determinants of infectious pancreatic necrosis virus virulence and cell culture adaptation. *J Virol* **79**(16)**,** 10289-99.

Strauss, J. H., and Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* **58**(3)**,** 491-562.

Taksdal, T., Stangeland, K., and Dannevig, B. H. (1997). Induction of infectious pancreatic necrosis (IPN) in Atlantic salmon Salmo salar and brook trout Salvelinus fontinalis by bath challenge of fry with infectious pancreatic necrosis virus (IPNV) serotype Sp. *Diseases of Aquatic Organisms* **28**(1)**,** 39-44.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**(7)**,** RESEARCH0034.

Villanueva, R. A., Galaz, J. L., Valdes, J. A., Jashes, M. M., and Sandino, A. M. (2004). Genome assembly and particle maturation of the birnavirus infectious pancreatic necrosis virus. *J Virol* **78**(24)**,** 13829-38.

Villoing, S., Bearzotti, M., Chilmonczyk, S., Castric, J., and Bremont, M. (2000). Rainbow trout sleeping disease virus is an atypical alphavirus. *J Virol* **74**(1)**,** 173-83.

Weber, S., Fichtner, D., Mettenleiter, T. C., and Mundt, E. (2001). Expression of VP5 of infectious pancreatic necrosis virus strain VR299 is initiated at the second in-frame start codon. *J Gen Virol* **82**(Pt 4)**,** 805-12.

Welsh, M., Weston, J., Borghmans, B. J., Mackie, D., Rowley, H., Nelson, R., McLoughlin, M., and Todd, D. (2000). Biochemical characterization of salmon pancreas disease virus. *J Gen Virol* **81**(Pt 3)**,** 813-20.

Weston, J., Villoing, S., Bremont, M., Castric, J., Pfeffer, M., Jewhurst, V., McLoughlin, M., Rodseth, O., Christie, K. E., Koumans, J., and Todd, D. (2002). Comparison of two aquatic alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection. *Journal of Virology* **76**(12)**,** 6155-6163.

Weston, J. H., Welsh, M. D., McLoughlin, M. F., and Todd, D. (1999). Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, Salmo salar L. *Virology* **256**(2)**,** 188-195.

White, D. E., and Fenner, F. J. (1994). Medical Virology, Fourth Edition *Publisher: Academic Press*.

White, L. J., Wang, J. G., Davis, N. L., and Johnston, R. E. (2001). Role of alpha/beta interferon in Venezuelan equine encephalitis virus pathogenesis: effect of an attenuating mutation in the 5' untranslated region. *J Virol* **75**(8)**,** 3706-18.

Wolf, K., Snieszko, S. F., Dunbar, C. E., and Pyle, E. (1960). Virus Nature of Infectious Pancreatic Necrosis in Trout. *Proceedings of the Society for Experimental Biology and Medicine* **104**(1)**,** 105-108.

Wood, E. M., Snieszko, S. F., and Yasutake, W. T. (1955). Infectious Pancreatic Necrosis in Brook Trout. *Archives of Pathology* **60**(1)**,** 26-28.

Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980). Cell death: the significance of apoptosis. *Int. Rev. Cytol.***,** 68:251–306.

Xu, H. T., Si, W. D., and Dobos, P. (2004). Mapping the site of guanylylation on VP1, the protein primer for infectious pancreatic necrosis virus RNA synthesis. *Virology* **322**(1)**,** 199-210.

Zhang, X., Ding, L., and Sandford, A. J. (2005). Selection of reference genes for gene expression studies in human neutrophils by real-time PCR. *BMC Mol Biol* **6**(1)**,** 4.