Necrosis Pancreatic Infectious Virus does not block 701-STAT1 (α/β) tyrosine in *Oncorhynchus mykiss* (Salmoniformes: Salmonidae).

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RESUMEN

El virus pancreático necrótico infeccioso no bloquea la tirosina 701-STAT1 (α / β) en *Oncorhynchus mykiss* (Salmoniformes: Salmonidae).

El virus de la Necrosis Pancreática infecciosa (IPNV) es un importante patógeno que afecta principalmente salmónidos. El tipo de interferón I alfa cumple un rol crucial como la primera línea de defensa contra la infección de IPNV. La activación de IFN-I(α) induce el señalamiento de la ruta JAK-STAT, uniendo sus receptores en una rápida fosforilación de STATs, un paso crítico para la translocación al núcleo induciendo los genes estimulados por el Interferón (ISGs). La relación entre el nivel de infectividad de las cepas de IPNV y la ruta de señalamiento de IFN es aun pobremente entendido. Nuestro propósito fue investigar si la ruta de señalamiento del IFN-I(α) es afectada por el nivel de infectividad de distintas cepas. Usamos dos aislados de IPNV (VR-299 y Sp) para infectar células RTG-2. El RNA total fue aislado usando un kit comercial para determinar la expresión de la proteína VP2 a nivel de mRNA de VP2 e IGS mediante qRT-PCR. Análisis de Western Immunoblot fue realizado para determinar el nivel de fosforilación de la proteína STAT1 (α/β) en el residuo tirosina 701en las células infectadas. La cepa de mayor virulencia no está asociada con un mayor aumento del efecto del bloqueo del señalamiento del interferón. Asimismo, la activación de la Y701-STAT1 (α/β) fue significativamente incrementada en las células infectadas con el virus del serotipo Sp, comparadas con el virus del serotipo VR-299, indicando que IPNV inhibe la ruta de señalamiento de IFN. IPNV no bloquea la fosforilación de la tirosina 701 STAT1 (α/β) estimulada por IFN-I(α), contrario como lo hacen otros virus de RNA..

Palabras claves: IPNV, fosforilación, 701-Tirosina STAT1 (α/β), trucha arcoíris.

ABSTRACT

Necrosis Pancreatic Infectious Virus does not block 701-STAT1 (α/β) tyrosine in Oncorhynchus mykiss (Salmoniformes: Salmonidae). Infectious pancreatic necrosis virus (IPNV) is a pathogen important that affects predominantly salmonids. The type I interferon alpha system has a crucial role in the first line of defense against IPNV infection. IFN-I(α) activation triggers the signaling pathway JAK-STAT, binding to their receptors results in the rapid phosphorylation of STATs a critical step for the nuclear translocation to induce the interferon stimulated genes (ISGs). The relationship between infectivity level of IPNV strain and pathway signaling of IFN is yet poorly understood. Our purpose was to investigate if the IFN-I(α) signaling

pathway is affected by IPNV strains of different infectivity levels. We used two IPNV isolated (VR-299 and Sp) to infect RTG-2 cells. Total RNA was isolated using the commercial kit for determine to VP2 expression and ISGs using qRT-PCR. Western Immunoblotting analysis was carried out for determine the 701 STAT1(α/β) phosphorylation into infected cells. Hence, a higher virulence strain is not associated with a greater blocking effect for interferon signaling. Furthermore, the activation of Y701-STAT1 (α/β) was significantly increased in serotype Sp virus infected cells compared with serotype VR-299 virus infected cells, indicating that IPNV inhibits IFN signaling pathway. As concluded, IPNV does not block the phosphorylation of 701-tyrosine STAT1 (α/β) stimulated by IFN-I(α), contrary to other RNA viruses.

Key words: IPNV, phosphorylation, 701-Tyrosyne STAT1 (α/β), rainbow trout.

Introduction

Infectious pancreatic necrosis virus (IPNV) is an aquabirnavirus member of the Birnaviridae family that causes an emerging disease which affects predominantly salmonids (OIE 2006; Smail et al., 2006). The clinical disease and the percentage of mortality depend on some factors such as water temperature, strain and infection pathway (Wolf, 1988; Arguedas et al., 2015). The virus produced a mortality ranged from 90 to 100% in rainbow trout (Oncorhynchus mykiss) with 1-4 month of age (McAllister & Bebak, 1997); although mortality levels can varey considerably, partly due to strain virulence variation (Santi et al., 2004). Two segments of double-stranded RNA (dsRNA), that encodes five viral proteins, composing the IPNV genome. The segment A encodes VP2, VP3, VP4 and VP5; while B encodes only VP1. Studies have shown that residues in positions 217 and 221 are key for determining the virulence of serotype Sp strains (Song et al., 2005). Virulent strains have a combination of threonine (Thr) and alanine (Ala) in positions 217 and 221, respectively (T217 A221), while strains of intermediate virulence carry proline P217 A221. Strains with (T217; T221) and T217 with P217 are avirulent. During IPNV infection, interferon type I (IFN-I(α)) binding to their receptors results in the rapid autophosphorylation (Skjesol et al., 2010) and the activation of the receptor associated to TYK2 and JAKs (Silvennoinen et al., 1993; Kotenko et al., 2003), which in turn regulate the phosphorylation and STATs activation in response to interferon (Darnell et al., 1994; Stark et al., 1998). Randall and Goodbourn (2008) observed in mammals that Y-701-STAT1 phosphorylation induce the binding of a STAT protein to other STAT, forming either a homodimer or a heterodimer which considered a critical step for the nuclear translocation. Hoeve et al. (2002) reported in HeLa cells and in murines that, TC-45 phosphatase is responsible for the dephosphorylation of STAT1 protein Y-701. In the nucleus, the STAT dimer binds to interferon stimulated response elements (ISRE) promoter to induce the mRNA expression of interferon stimulated genes (ISGs) against viral infection in teleost (Robertsen et al., 2003; Zhou et al., 2007; García et al., 2011). Also, several studies have demonstrated the action of ISGs in vitro and in vivo (Kinkelin & Dorson, 1973; Eaton 1990; Robertsen 2006; Verrier et al., 2011), such as Mx, 2-5 Oligo Adenylate Sintetase (2'-5' OAS), antiviral protein viperine (Vig-1), RNA-dependent Protein Kinase (PKR) among others (Sen, 2001; Platanias, 2005). The JAK-STAT pathway and transcription factors associated with the family of STAT proteins have been poorly studied in fish (Jørgensen et al., 2007; Zhou et al., 2007). Although fish genomes contain all mammals JAK-STAT pathway component keys, including JAK1, TYR2, STAT1, STAT2 and IRF9 (Leu et al., 2000; Stein et al., 2007; Collet et al., 2009; Sun et al., 2009; Shi et al., 2012). In the animal kingdom, the signaling pathway JAK - STAT is a powerful defense mechanism, some viruses may affect this pathway by the usage of various strategies which blocks or decreases the antiviral response (Randall & Goodbourn, 2008). For example, Ebola virus blocks the induction of interferon regulatory factor (IRF) (Harcourt, 1999), hepatitis C virus (HCV) blocks the interferon stimulated gene factor 3 (ISGF3) (Heim et al., 1999). Dengue virus serotype 2 inhibits the expression of STAT2, West Nile virus (WNV) blocks the tyrosine phosphorylation of STAT1 701 (Liu et al., 2005), and encephalitis Japanese virus (JEV) blocks the phosphorylation of Tyk2 and STAT activation (Ling et al., 2006). Likewise, studies have reported that Sendai virus (SeV) can interact with STAT1 inhibiting tyrosine phosphorylation of STAT induced by IFN-I(α) (Garcin et al., 1999; Gotoh et al., 1999; Komatsu et al., 2000). However, data concerning STAT1 activation in fish through the JAK-STAT pathway are limited. Assuming that STAT1 is a key cellular protein, which activates ISGs and it has a function like transcription factor subsequent to interferon type I receptor binding; our purpose was investigate if the IFN-I(α) signaling pathway is affected by IPNV strains of different infectivity levels.

Methodology

Viruses and cells

We used two IPNV isolated, a virus VR-299 serotype which is considered as avirulent strain (Gen Bank Access GU072914) containing Alanine 217 and Threonine 221 residues (A217 Thr221). Additionally, a virus Sp serotype of moderate virulence strain (Gen

Bank Access GU072916) contain Proline 217 and Alanine 221 (P217 A221) (Ortega et al., 2011). Both viruses were considered important due to difference clinical-pathology history showed in fish affected and mortality rates caused in salmon farms in southern of Chile. Viruses were replicated by inoculation in bottles of 500 mL in Chinook salmon embryo cells (CHSE-214) with 90% confluence maintained in minimal essential medium (MEM) to 2% of fetal bovine serum (FBS) and once reached an extensive cytopathic effect (EPC) viruses were tittered, using microplate method (Reed and Muench, 1938). Then, viruses were used for cell infection.

Cellular infection: (VP2 expression and ISGs)

RTG-2 cells (Rainbow trout Gonad) in 6-wells plates at 15°C with L-15 medium with 2% FBS (Gibco, Invitrogen Corp. Carlsbad, California) were infected in triplicate with IPNV strains at MOI=0.1(Multiplicity Of Infection). Afterward infection, the incubation continued at the same temperature until it was interrupted at 4, 8, 24, 36 and 60 hours' post-infection (hpi) when the supernatant was eliminated.

RNA extraction and RT-PCR

Total RNA was isolated using the commercial kit (E.Z.N.ATM Total RNA Kit I, Omega-Biotek) and according to the manufacturer's instructions. Then, 9,4 µL of RNA was incubated using a thermocycler (Labnet Multigene Gradient) at 37°C for 30 minutes (min), and was treated with DNase (Promega Cat. #M6101), mixed with 1µL of RQ1 RNase-Free DNase and 1µL RQ1 DNase 10X Reaction Buffer per sample. Immediately, 1µL of stop solution was added, and the samples were incubated at 65°C for 10 min. As a start the RT-PCR for the cells was conducted, immediately a first mix containing 1µL dNTPs 10 mM, 1,6 µL Oligo dT15mer was added to the samples, and were incubated at 60°C for 10 min. Then, samples were incubated on ice. Subsequently, a second mix containing 4 µL of M-MLV Reverse Transcriptase 5X Reaction Buffer (Promega M531A), 0,5 µL of RNasin 40U/µL (Promega) and 0,5 µL M-MLV Reverse Transcriptase 200U/µL (Promega) were added. Then, samples were incubated in a thermocycler, programmed to complete a cycle of 42°C for 60 min x40 cycles, and finally at 70°C for 10 min for extension, samples were maintained at 4°C. For viral RT-PCR described as follow: a first mix contain 2 µL dNTPs 10 mM, 1 µL Random primers were added to the samples, and incubated at 60°C for 10 min. After samples were incubated on ice. Subsequently, a second mix containing 4 µL of M-MLV Reverse Transcriptase 5X Reaction Buffer (Promega M531A), 0,5 µL of RNasin 40U/µL (Promega) and 0,5 µL M-MLV Reverse Transcriptase 200U/µL (Promega) were added. Reactions were carried in a thermocycler, programmed to complete a cycle of 42°C for 60 min x40 cycles and finally at 70°C for 10 min for extension, samples were maintained at 4°C.

Real-Time PCR (qRT-PCR)

To determine IFN-I(α), STAT-1(α) and Mx-1 gene expression, cDNA was amplified with a Step OneTM Real Time PCR system Thermal Cycling Block (Applied Biosystems) using the SYBR®Green method. PCR amplification was performed in individual wells of a 48-well optical plate, mixing 2 µL of cDNA, 1 µL primer Forward, 1 µL primer Reverse, 1 µL DEPC water and 5µL of SYBR®Green PCR Master Mix (Cat. #4344463), for a final volume of 10 µL per sample. The standard cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. All samples were analyzed in triplicate. The efficiency of the primers was tested using serial dilutions of a known initial template (101-1010) to produce a standard curve. Relative quantification of the amplified gene products was calculated by 2-ΔΔCt method (Livak & Schmittgen, 2001). To determine viral replication (VP2 gene expression), the VP2 cycle threshold (Ct) values were converted into expression values normalized against the endogenous reference gene using the statistical standard curve method as described in the Applied Biosystems User Manual. Housekeeping gene elongation factor 1 alpha (ELF-1(α)) was used for RTG-2 cell genes and IPNV/VP2, as the endogenous reference gene. Specific primer sequences are documented in Table 1.

Western Immunoblotting analysis for STAT1(α/β) phosphorylation

RTG-2 fibroblast cell line were cultured in 6-well plates at 15°C containing L-15 medium (Gibco, Invitrogen Corp., Carlsbad, California) supplemented with 10% fetal bovine serum per well. Before proceeding to experiment, cells were starved overnight in a 2% FBS medium in order to lower basal protein phosphorylation, and then were infected in triplicate with IPNV strains at MOI=0,1. After infection at 15°C the incubation continued at the same temperature until it was interrupted at 0 min, 1, 4, 8, 12 and 24 hpi. Subsequently, cells were lysated using 300 µL of lysis buffer (500 mM Tris-HCl pH 7,5, 150 mMNaCl, 1mM EDTA, 1 mM EGTA, 0,25% sodium deoxycholate, 1 mM PMSF,1 mM p-nitrofenilfosfato, 1% NP-40, protease and phosphatase inhibitor mixture to 1%) and incubated on ice for 5 min, and then scraped.

Table 1. Primers used for Real time (*q*RT-PCR) during the mRNA expression analysis of antiviral genes and protein VP2 -IPNV in RTG-2 infected cell with strain of different virulence.

Name	Sequence 5' ⟶3'	Product size (bp)[a]	GAN[b]/or Reference (Source)
IFN-I(*α*)	F: CCTGCCATGAAACCTTGAGAAGA R:TTTCCTGATGAGCTCCCATGC	108	Fj184371.1
STAT1(*α*)	F: CGGGCCCTGTCACTGTTC R: GGCATACAGGGTGTCTCT	68	NM_001124707.1
Mx-1	F: AGCTCAAACGCCTGATGAAG R: ACCCCACTGAAACACACCTG	142	NM_001171901
ELF-1(α)	F: ACCCTCCTCTTGGTCGTTTC R: TGATGACACCAACAGCAACA	63	NM_001124339.1
VP2	WB1-CCGCAACTTACTTGAGATCCATTATGC WB2-CGTCTGGTTCAGATTCCACCTGTAGTG	206	Williams et al. (1999)

[a] Product size PCR. bp (base pairs).
[b] Sequences to forward (F), reverse (R) are given according to access number in Genbank. (GAN) or reference.

The resultant proteins were centrifuged at 14,000 × g for 10 min at 4°C and quantified by Bradford's methods using BSA (20mg/mL) as standard. A measure of 100 µg of protein was resolved in 12% SDS-PAGE and transferred to a nitrocellulose membrane for 2 hours (hrs), at 200 mA. The membrane was then blocked with buffer (1 × TBS, 0,1% Tween-20 and 5% nonfat dry milk) for 2 hrs at room temperature, washed, and incubated overnight at 4°C with an antiphospho-STAT1(α/β) (Tyr701) monoclonal antibody at a dilution of 1:500 (Cell signaling, Beverly, MA, USA, cat # 7649). The membrane was washed and then incubated with goat anti-rabbit IgG-HRP-conjugated antibody secondary at a dilution 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hrs, then visualized using an enhanced chemiluminescence (ECL) system (Perkin-Elmer, USA). Molecular weight of phosphoprotein was determined based on the mobility of prestained standards of known molecular weight. The primary antibodies were removed by incubation with stripping solution (100 mM 2-mercaptoethanol; 2% SDS; 62,5 mM Tris-HCl, pH 6,7) for 30 min at 50°C with agitation, followed by several washes with TBS-Tween 0,1%). Each membrane was reused with anti-actin antibody at a dilution of 1:1000 (Sigma-Aldrich, USA, cat # A4700), using a procedure similar to the described above, but as secondary antibody a Goat anti-Mouse IgG (H+L) HRP conjugate (Thermo Scientific Pierce) at a dilution of 1:2000. The intensity of each band was analyzed using the Software Image J and was normalized to actin. We used (100µL) of cell's secretome infected with each serotype as a positive control, and no infected cells as negative control.

Statistical Analysis

The expression gene means and phosphorylation rates resulting were compared by Student t-test in each time points. Also, were implemented comparison of the phosphorylation rates to the control group (cell no infected). Data were presented as mean ± SD. We performed Pearson's correlation (r) to determine the strength of association between genes expression. Anderson-Darlington normality test was used to know if the variables were normally distributed. Statistical analyses were performed using GraphPad Prism® 6 (GraphPad Software, Chicago, Inc, USA). The shown Experiment is a representative of three independent experiments, which generated reproducible data, means ± SD. The main differences of the group were considered statistically significant if the p-value was <0,05.

Ethical, conflict of interest and financial statements

The authors declare that they have fully complied with all ethical and legal requirements, both during the study and in the production of the manuscript; that there are no conflicts of interest of any kind; that all financial sources are fully and clearly stated in the acknowledgements section; and that they fully agree with the final edited version of the article. A signed document has been filed in the journal archives.

Results

IPNV replication

Serotype Sp protein expression (Sp-VP2) showed a transcript higher level from 4 to 36 hours post infection (hpi) ($P < 0,05$; Figure 1) when was compared with values obtained for (VR-299-VP2), except at 60 hpi where VP2-VR-299 mRNA was higher. However, the viral replication was increasing by both IPNV isolates after 4 hpi, although, the higher increase was showed by Sp serotype virus ($P < 0,05ab$).

On the other hand, the mRNA IFN-I(α) detected at 4 and 8 hpi in cells infected with VR-299 virus was significantly lower when was compared to the Sp Virus ($P < 0,05ab$). Additionally, the interferon expression values showed significant differences at 8 and 12 hpi ($P < 0,05$; Figure 2B), and values expression were subsequent irregulars for this case. However, the expression increase of VP2-VR-299 did not show a clear relationship with the expression of this cytokine. The STAT-1(α) mRNA in infected cells with serotype Sp virus showed a kinetic similar expression to the detected by interferon from 4 to 60 hpi (Figure 2C). However, registered values at 4 and 8 hpi were higher compared to the expressed values in infected cells with serotype virus VR-299 ($P < 0,05ab$). Likewise, mRNA STAT-1(α) in infected cells with latter serotype were higher at 24 and 60 hpi when was compared with serotype Sp virus. Moreover, STAT-1(α) mRNA showed a kinetic similar expression to the registered by IFN-I(α), showing increase and decrease in the same time points ($r = 0,85$, $p < 0,0001$). According to Mx-1 mRNA, a rapid expression was obtained at 4 hpi in infected cells with serotype Sp virus, although a significant expression decrease at 8, 12 and 36 hpi was found ($P < 0,05$; Figure 2E). Contrary, the Mx-1 transcript expressed at 4 hpi in infected cells with serotype VR-299 virus was lower, when was compared with obtained value in infected cells with serotype Sp virus. In addition, Mx-1 expression values (Figure 2F) were similar to the observed in the infected cells with serotype Sp virus in other time-points coinciding with an increase of viral replication.

Y701-STAT1(α/β) phosphorylation expressed in infected RTG-2 cells with IPNV

STAT1 (α/β) activation was induced in infected cells with both IPNV serotypes in early infection stage. However, in cells infected with serotype Sp virus the activation level showed a significant increase at 4, 8, 12 y 24 hpi (Figure 3A) compared to activation level registered in infected cells with serotype VR-299 virus ($P < 0,05ab$). Further, the Y701-STAT1(α/β) activation in infected cell with serotype Sp virus was increasing from zero minutes (0 hpi) until 12 hpi, showing a significant phosphorylation peaks at 12 and 24 hpi (2,7 and 1,36 fold respectively) compared to control group ($P < 0,05$). However, contrary, the level phosphorylation revealed in infected cell with serotype VR-299 virus did not show a significant difference in the same time-points ($P > 0,05$; Figure 3B), however, the phosphorylation peaks were at 12 and 24 hpi also (~ 0,175 fold in each time point), but the values were lower.

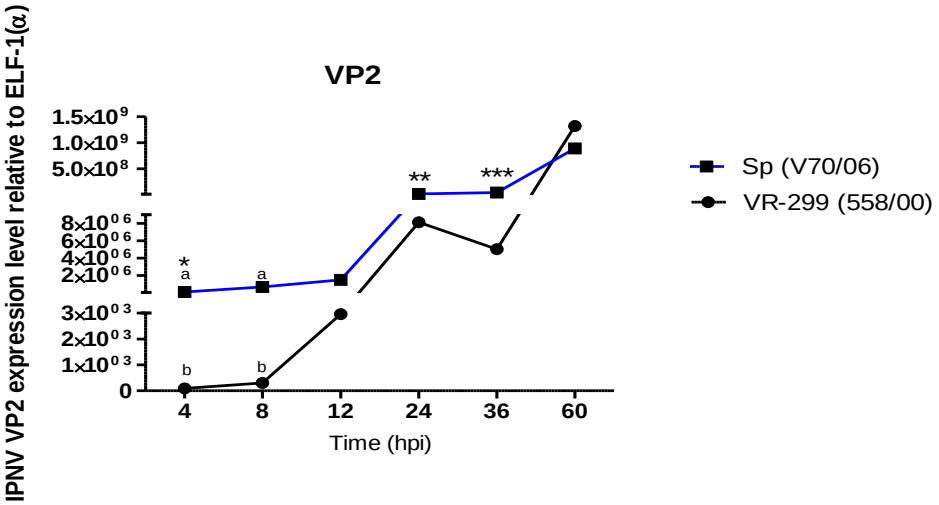

Figure. 1. Expression level of VP2 mRNA in RTG-2 cells infected with two IPNV serotypes of differences virulence. Sp (virulent strain) and VR-299 (avirulent strain). (MOI=0.1)

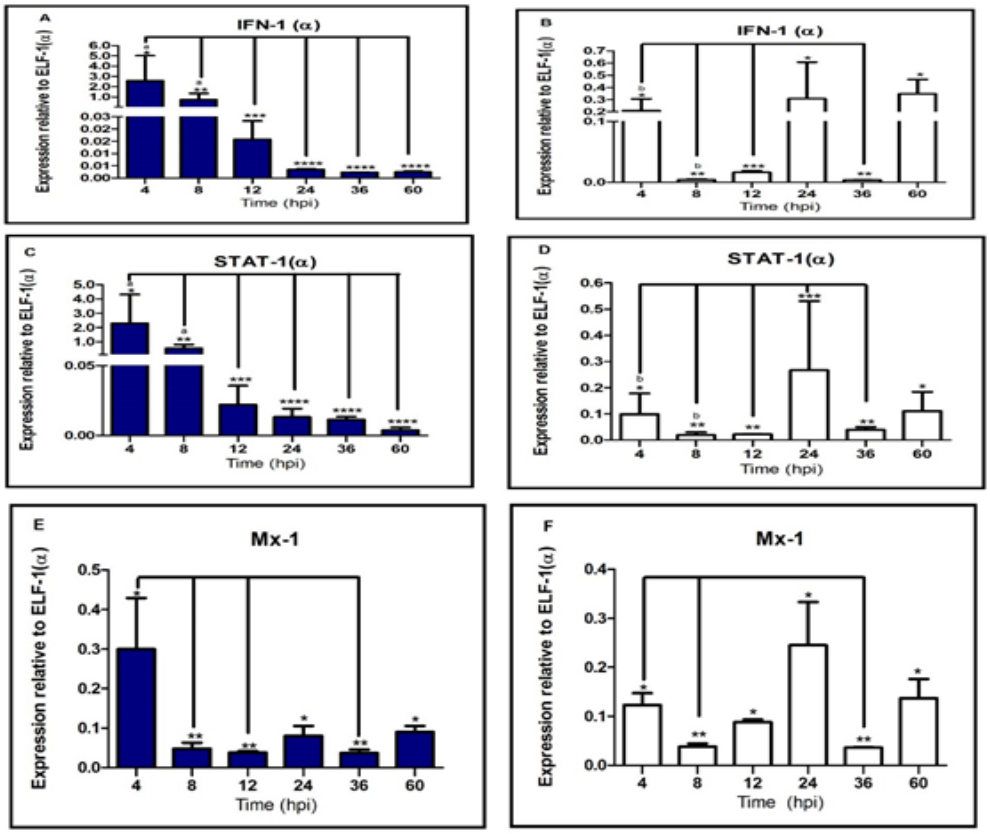

Figure. 2. Expression level of IFN-I(α), STAT-1(α) and Mx-1 mRNA in RTG-2 cells infected with two IPNV serotypes of differences virulence (MOI=0.1). Infected cell with serotype Sp (A, C, E) and cell with serotype VR-299 virus (B, D, F).

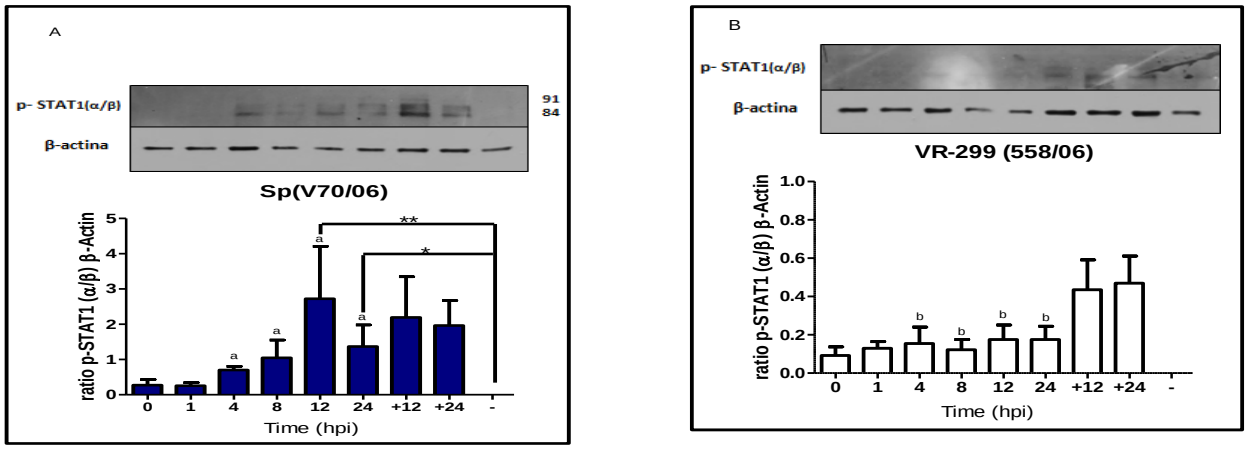

Figure. 3. Phosphorylation level of STAT1 (α/β) (pY701-STAT1 (α/β)) in RTG-2 cells infected with two IPNV serotypes of difference virulence (MOI=0.1). Infected cell with serotype Sp (A, C, E) and cell with serotype VR-299 virus (B, D, F).

Discussion

The IPNV infection induces the synthesis of genes involved in nonspecific immune response (Robertsen, 2008). Although, the relationship between the response level and the virus strain virulence is not clear. In the present study, we evaluated two IPNV serotypes to know if the infectivity level of the strains affects the signaling pathway of type I interferon alpha (IFN-I(α)) in RTG-2 cells. The experiment which evaluate the viral replication, indicates higher virulence of the Sp virus (Wolf, 1988; Dobos, 1995), which involved the Sp strains in cases of high mortality rates or severe clinical pictures (Santi et al., 2004; Santi et al., 2005). Sano et al. (1992), related to the

IPNV virulence with segment A, although recently was associated to the residues 217 and 221 of VP2 protein (Santi et al., 2004). Highly virulent isolates possess residues Thr217 and Ala221; moderate to low-virulence strains have Pro217 and Ala221; and strains containing Thr221 are almost avirulent, irrespective to the residue at position 217 (Song et al., 2005). Based on the above, strains used in this study corresponding to moderate virulence (Sp serotype) and avirulent (VR-299). Although, Smail et al. (2006) did not find mortality differences to compare a high virulent strain with a moderate virulence strain, suggesting that others factors associated to the strain or immune response possibly affect the infection findings (Ortega et al., 2011). In our study, we might hypothesize that higher viral replication before 24 hpi are associated with a greater penetration for serotype Sp virus, linked to the amino acids residue sequences of the hypervariable region of VP2 binding protein. In addition, this region might interact with cell receptors in a different way (Dobos 1995; Kuznar et al., 1995; Granzow et al., 1997). Earlier work demonstrated that peptide derived of Vp2 maturation of infectious bursal disease virus (IBDV) participates in the virus-cell entry suggesting that peptide 46 (pep 46) has a domain rich in proline (positions 458, 465, 469) that disrupt cell membrane and induces pores (Galloux et al., 2007). However, in our work we did not find differences between strains in VP2 for peptide 46 (results not shown), therefore, both viruses should behave similarly, so this peptide 46 is not implicated in viral replication difference between both serotypes. Therefore, viral replication differences might to be associated with other structural and functional aspects that have been implicated to IBDV replication (Da Costa et al., 2002).

Some proteases like IPNV-Vp4 protein have been considered as virulence factors; however, this finding is not been shown yet (Skjesol et al., 2009); additionally, the possible involvement of VP5 protein as a virulence factor has been discarded.

We demonstrated that, apparently others viral and cellular factors have influenced that serotype Sp virus show a higher replication. Interestingly the serotype VR-299 virus reached replication values higher to the Sp at 60 hpi. Studies by Kuznar et al. (1995) showed that at 10 hpi, viral RNA is detected and at 14 hpi mature particles were detected also, so this situation might be associated with a random value of viral multiplicity because at this time-points, more than two replication cycles have elapsed (Espinoza et al., 2000). IPNV infection induced the expression of IFN-I(α) that module an antiviral response stimulating the other gene effectors (Saint-Jean and Pérez-Prieto, 2007). Our results indicate that replication increase in both serotypes (Sp serotype virus higher than VR-299) was associated a decreased of IFN-I mRNA, suggesting that, inhibition of alpha interferon signaling is required for viral replication in early stages of infection. This finding is consistent with observations from (Skjesol et al., 2009) that described the ability of IPNV to reduce the interferon immune response; however, contrary to the hypothesized; the IFN-I(α) expression was higher in infected cells with serotype Sp virus, suggesting a lineal positive correlation between strain virulence and alpha interferon immune response.

Although a high expression of this cytokine might produce a negative effect in cells implicated in high mortality caused by the cell production of proteases and other proteins that contribute to cell damage (Hay & Kannourakis, 2002). In contrast to the outcomes in infected cells with VR-299 serotype virus (avirulent strain), the cellular response was attenuated. Additionally, to this study, earlier work demonstrated that IPNV inhibits the mechanism of interferon signaling (Robertsen, 2008). In relation to this, we showed an indirect relationship between VP2-IPNV expression and mRNA STAT-1(α) expression, associated with a decrease of interferon transcript simultaneously, explained by STAT-1(α) is a ISGs. However, our research also showed that higher virulence strain is not associated with a greater effect for blocking of alpha interferon signaling. This outcome can be explained by STAT-1(α) expression was higher at 8 hpi in infected cells with serotype Sp virus. Additionally, this latter finding is supported by higher STAT1(α/β) phosphorylation level induced by serotype Sp virus compared to serotype VR-299 virus between 4 and 24 hpi (phosphorylation peak at 12 hpi).

Additionally, we demonstrated that Mx-1 protein inhibit IPNV replication (Larsen et al., 2004; Jørgensen et al., 2007). However, Mx-1 transcript was down-regulated simultaneously, the virus replication increased. Possibly, because RTG-2 cells were not priory stimulated with IFN-I or Poly: IC (Skjesol et al., 2009). Interestingly, the Mx-1 transcript level between both strains was similar to other study (Ortega et al., 2011), which reported virulence strain does not affect the Mx expression; we suggest that both strains has a similar antiviral sensitivity against the interferon response and ISGs such as Mx protein. A Mx protein positive effect against infection IPNV possibly is mediated by other factors such as isoform and amount of protein, cells type, infection temperature among others (Arguedas et al. 2015). The Y-701 STAT1(α/β) phosphorylation mediates rapid and robust activity and expression specific transcriptional of genes for the activating of cytokines and cell factors growth (Decker et al., 2002; Skjesol et al., 2010). Our results showed that STAT1(α/β) -pY701 level was significantly

increased in infected cells serotype Sp virus, determined by a significant increase of IFN-I(α) expression at an early point after infection (4 and 8 hpi) compared to values observed in infected cell serotype VR-299 virus. This suggest that, 701 STAT1(α/β) tyrosine was activated by interferon type I(α) in trout, but not by intracellular type (iIFN-Ib) that was reported for the first time in vertebrates (rainbow trout), not showing biological activity on STAT1 and STAT2 phosphorylation (Chang et al., 2013). The alpha interferon response against IPNV infection induced 701-STAT1(α/β) phosphorylation with both serotypes. Fascinatingly, this observation illustrate that IPNV does not inhibit the phosphorylation of 701-tyrosine-STAT1(α/β) stimulated by IFN-I(α), contrary to other RNA viruses (Horvath, 2004; Randall & Goodbourn, 2008). Nevertheless, the level Y701-STAT1(α/β) activation is directly correlated with the strains IPNV virulence. Activation of STAT1(α/β) in mammals contributes to the maximum transcriptional activation and apoptosis (Sironi & Ouchi, 2004; Thomas et al., 2004; Townsend et al., 2004).

Apoptosis previously documented by IPNV infected fish both in vitro and in vivo independently strain virulence (Hong et al., 1999; Espinoza et al., 2005; Ortega et al., 2014). Hence, our results of higher STAT1(α/β) activation found in infected cell with serotype Sp virus indicates apparently that apoptosis mechanism was required for rapid elimination of infected cell, such as high cellular response to the strain virulence. Additionally, the STAT-1(α) and IFN-I(α) expression were down-regulated, that might be associated to a lower quantity of live cells. Contrary, the up-regulation gene expression found in infected cell with serotype VR-299 virus at 24 and 60 hpi which could be a consequence of a higher cell's ability to establish an antiviral effect. However, information associated with STAT1(α/β) phosphorylation after IPNV infection in teleost is limited and the apoptosis depend completely on the Y-701STAT1 (α/β) phosphorylation and it is an interesting question that needs to be addressed in future studies. Our data indicates a negative effect by IPNV on signaling alpha interferon and ISGs independently to the strain virulence. IPNV does not block the phosphorylation 701STAT1 (α/β) tyrosine contrary to other RNA viruses. Further studies are clearly needed in order to identify others molecular mechanisms how IPNV inhibits signaling pathway alpha interferon.

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References

Arguedas Cortés, D., Romero Zuñiga, A. P., Enriquez Sais, R., Martínez Castañeda, J. S., & Ortega Santana, C. (2015). Effect of temperature on the expression of IFN-1 (α), STAT-1 and Mx-1 genes in *Oncorhynchus mykiss* (Salmoniformes: Salmonidae) exposed with the virus of the infectious pancreatic necrosis (IPNV). *Revista de Biología Tropical*, *63*(2), 559-569.

Chang, M. X., Zou, J., Nie, P., Huang, B., Yu, Z., Collet, B., & Secombes, C. J. (2013). Intracellular interferons in fish: a unique means to combat viral infection. *PLoS pathogens*, *9*(11), e1003736. doi: 10.1371/journal.ppat.1003736

Collet, B., Ganne, G., Bird, S., & Collins, C. M. (2009). Isolation and expression profile of a gene encoding for the Signal Transducer and Activator of Transcription STAT2 in Atlantic salmon (*Salmo salar*). *Developmental & Comparative Immunology*, *33*(7), 821-829. doi: 10.1016/j.dci.2009.01.007

Collet, B., Munro, E. S., Gahlawat, S., Acosta, F., Garcia, J., Roemelt, C., ... & 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 Immunology*, *22*(1-2), 44-56. doi: 10.1016/j.fsi.2006.03.011

Da Costa, B., Chevalier, C., Henry, C., Huet, J. C., Petit, S., Lepault, J., ... & Delmas, B. (2002). The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of pVP2. *Journal of virology*, *76*(5), 2393-2402. doi: 10.1128/jvi.76.5.2393-2402.2002

Darnell, J. E., Kerr, I. M., & Stark, G. R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*, *264*(5164), 1415-1421. doi: 10.1126/science.8197455

De Kinkelin, P., & Dorson, M. (1973). Interferon production in rainbow trout (*Salmo gairdneri*) experimentally infected with Egtved virus. *Journal of General Virology*, *19*(1), 125-127. doi: 10.1099/0022-1317-19-1-125

Decker, T., Stockinger, S., Karaghiosoff, M., Müller, M., & Kovarik, P. (2002). IFNs and STATs in innate immunity to microorganisms. *The Journal of clinical investigation*, *109*(10), 1271-1277. doi: 10.1172/JCI15770

Dobos, P. (1995). The molecular biology of infectious pancreatic necrosis virus (IPNV). *Annual Review of Fish Diseases*, *5*, 25-54. doi.org/10.1016/0959-8030(95)00003-8

Eaton, W. D. (1990). Anti-viral activity in four species

of salmonids following exposure to poly inosinic: cytidylic acid. *Diseases of Aquatic Organisms*, *9*(3), 193-198. doi: 10.3354/dao009193

Espinoza, J. C., Cortés-Gutierrez, M., & Kuznar, J. (2005). Necrosis of infectious pancreatic necrosis virus (IPNV) infected cells rarely is preceded by apoptosis. *Virus research*, *109*(2), 133-138. doi: https://doi.org/10.1016/j.virusres.2004.10.014

Espinoza, J.C., Hjalmarsson, A., Everitt, E., & Kuznar, J. (2000). Temporal and subcellular localization of infectious pancreatic necrosis virus structural proteins. *Archives of Virology 145*(4): 739-748. doi: https://doi.org/10.1007/s007050050667

Galloux, M., Libersou, S., Morellet, N., Bouaziz, S., Da Costa, B., Ouldali, M., ... & Delmas, B. (2007). Infectious bursal disease virus, a non-enveloped virus, possesses a capsid-associated peptide that deforms and perforates biological membranes. *Journal of Biological Chemistry*, *282*(28), 20774-20784. doi: 10.1074/jbc.M701048200

García, I., Galiana, A., Falcó, A., Estepa, A., & Perez, L. (2011). Characterization of an infectious pancreatic necrosis (IPN) virus carrier cell culture with resistance to superinfection with heterologous viruses. *Veterinary microbiology*, *149*(1-2), 48-55. doi: 10.1016/j.vetmic.2010.10.017.

Garcin, D., Latorre, P., & Kolakofsky, D. (1999). Sendai virus C proteins counteract the interferon-mediated induction of an antiviral state. *Journal of Virology*, *73*(8), 6559-6565.

Gotoh, B., Takeuchi, K., Komatsu, T., Yokoo, J., Kimura, Y., Kurotani, A., ... & Nagai, Y. (1999). Knockout of the Sendai virus C gene eliminates the viral ability to prevent the interferon-α/β-mediated responses. *FEBS letters*, *459*(2), 205-210. doi: 10.1016/s0014-5793(99)01241-7

Granzow, H., Weiland, F., Fichtner, D., & Enzmann, P. J. (1997). Studies of the ultrastructure and morphogenesis of fish pathogenic viruses grown in cell culture. *Journal of Fish Diseases*, *20*(1), 1-10. doi: https://doi.org/10.1046/j.1365-2761.1997.00267.x

Harcourt, B. H., Sanchez, A., & Offermann, M. K. (1999). Ebola virus selectively inhibits responses to interferons, but not to interleukin-1β, in endothelial cells. *Journal of virology*, *73*(4), 3491-3496.

Hay, S., & Kannourakis, G. (2002). A time to kill: viral manipulation of the cell death program. *Journal of General Virology*, *83*(7), 1547-1564. doi: 10.1099/0022-1317-83-7-1547

Heim, M. H., Moradpour, D., & Blum, H. E. (1999). Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. *Journal of Virology*, *73*(10), 8469-8475.

Hoeve, J., de Jesus Ibarra-Sanchez, M., Fu, Y., Zhu, W., Tremblay, M., David, M., & Shuai, K. (2002). Identification of a nuclear Stat1 protein tyrosine phosphatase. *Molecular and cellular biology*, *22*(16), 5662-5668. doi: 10.1128/mcb.22.16.5662-5668.2002

Hong, J. R., Hsu, Y. L., & Wu, J. L. (1999). Infectious pancreatic necrosis virus induces apoptosis due to down-regulation of survival factor MCL-1 protein expression in a fish cell line. *Virus research*, *63*(1-2), 75-83. doi: 10.1016/s0168-1702(99)00060-x

Horvath, C. M. (2004). Weapons of STAT destruction. *European journal of biochemistry*, *271*(23-24), 4621-4628. doi: https://doi.org/10.1111/j.1432-1033.2004.04425.x

International Office of Epizootics. Aquatic Animal Health Standards Commission. (2006). *Manual of diagnostic tests for aquatic animals*. Office International des Epizooties.

Jensen, I., & 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 immunology*, *13*(3), 221-241. doi: https://doi.org/10.1006/fsim.2001.0397

Jørgensen, J. B., Johansen, A., Hegseth, M. N., Zou, J., Robertsen, B., Collet, B., & Secombes, C. J. (2007). A recombinant CHSE-214 cell line expressing an Mx1 promoter–reporter system responds to both interferons 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. doi: 10.1016/j.fsi.2007.07.008

Komatsu, T., Takeuchi, K., Yokoo, J., Tanaka, Y., & Gotoh, B. (2000). Sendai virus blocks alpha interferon signaling to signal transducers and activators of transcription. *Journal of virology*, *74*(5), 2477-2480. doi: 10.1128/JVI.74.5.2477-2480.2000

Kotenko, S. V., Gallagher, G., Baurin, V. V., Lewis-Antes, A., Shen, M., Shah, N. K., ... & Donnelly, R. P. (2003). IFN-λs mediate antiviral protection through a distinct class II cytokine receptor complex. *Nature immunology*, *4*(1), 69. doi: 10.1038/ni875

Kuznar, J., Soler, M., Farias, G. I. L. D. A., & Espinoza, J. C. (1995). Attachment and entry of infectious pancreatic necrosis virus (IPNV) into CHSE-214 cells. *Archives of virology*, *140*(10), 1833-1840. doi: 10.1007/bf01384345

Larsen, R., Røkenes, T. P., & Robertsen, B. (2004). Inhibition of infectious pancreatic necrosis virus replication by Atlantic salmon Mx1 protein. *Journal of virology*, *78*(15), 7938-7944. doi: 10.1128/JVI.78.15.7938-7944.2004

Leu, J. H., Yan, S. J., Lee, T. F., Chou, C. M., Chen, S.

T., Hwang, P. P., ... & Huang, C. J. (2000). Complete genomic organization and promoter analysis of the round-spotted pufferfish JAK 1, JAK 2, JAK 3, and TYK 2 genes. *DNA and cell biology*, *19*(7), 431-446. doi: 10.1089/10445490050085924

Lin, R. J., Chang, B. L., Yu, H. P., Liao, C. L., & Lin, Y. L. (2006). Blocking of interferon-induced Jak-Stat signaling by Japanese encephalitis virus NS5 through a protein tyrosine phosphatase-mediated mechanism. *Journal of virology*, *80*(12), 5908-5918. doi: 10.1128/JVI.02714-05

Liu, W. J., Wang, X. J., Mokhonov, V. V., Shi, P. Y., Randall, R., & Khromykh, A. A. (2005). Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. *Journal of virology*, *79*(3), 1934-1942. doi: 10.1128/JVI.79.3.1934-1942.2005

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, *25*(4), 402-408. doi: 10.1006/meth.2001.1262

McAllister, P. E., & Bebak, J. (1997). Infectious pancreatic necrosis virus in the environment: relationship to effluent from aquaculture facilities. *Journal of Fish Diseases*, *20*(3), 201-207. doi: 10.1046/j.1365-2761.1997.00297.x

Ortega, C., Rodríguez, S., Ana, I., Romero, A., Monrás, M., & Enríquez, R. (2011). Evaluation of the level of Mx3 protein synthesis induced by infectious pancreatic necrosis virus (IPNV) strains of different infectivity. *Veterinary immunology and immunopathology*, *141*(3-4), 190-200. doi: 10.1016/j.vetimm.2011.02.022

Ortega, S., Rodríguez, S., Espinoza, J. C., Kuznar, J., Romero, A., & Enríquez, R. (2014). Relationship between apoptosis and the BH2 domain sequence of the VP5 peptide of infectious pancreatic necrosis virus. *Revista MVZ Córdoba*, *19*(1), 3990-4002. doi: https://doi.org/10.21897/rmvz.119

Platanias, L. C. (2005). Mechanisms of type-I-and type-II-interferon-mediated signalling. *Nature Reviews Immunology*, *5*(5), 375. doi: 10.1038/nri1604

Randall, R. E., & Goodbourn, S. (2008). Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *Journal of General Virology*, *89*(1), 1-47. doi: 10.1099/vir.0.83391-0

Reed, L. J., & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *American journal of epidemiology*, *27*(3), 493-497. doi: https://doi.org/10.1093/oxfordjournals.aje.a118408

Robertsen, B. (2006). The interferon system of teleost fish. *Fish & shellfish immunology*, *20*(2), 172-191. doi: 10.1016/j.fsi.2005.01.010

Robertsen, B. (2008). Expression of interferon and interferon-induced genes in salmonids in response to virus infection, interferon-inducing compounds and vaccination. *Fish & shellfish immunology*, *25*(4), 351-357. doi: 10.1016/j.fsi.2008.02.004

Robertsen, B., Bergan, V., Røkenes, T., Larsen, R., & Albuquerque, A. (2003). Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. *Journal of Interferon & Cytokine Research*, *23*(10), 601-612. doi: 10.1089/107999003322485107

Saint-Jean, S. R., & Pérez-Prieto, S. I. (2007). Effects of salmonid fish viruses on Mx gene expression and resistance to single or dual viral infections. *Fish & shellfish immunology*, *23*(2), 390-400. doi: 10.1016/j.fsi.2006.11.012

Sano, M., Okamoto, N., Fukuda, H., Saneyoshi, M., & 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. doi: 10.1111/j.1365-2761.1992.tb00666.x

Santi, N., Song, H., Vakharia, V. N., & Evensen, Ø. (2005). Infectious pancreatic necrosis virus VP5 is dispensable for virulence and persistence. *Journal of virology*, *79*(14), 9206-9216. doi: 10.1128/JVI.79.14.9206-9216.2005

Santi, N., Vakharia, V. N., & Evensen, Ø. (2004). Identification of putative motifs involved in the virulence of infectious pancreatic necrosis virus. *Virology*, *322*(1), 31-40. doi: 10.1016/j.virol.2003.12.016

Sen, G. C. (2001). Viruses and interferons. *Annual Reviews in Microbiology*, *55*(1), 255-281. doi: https://doi.org/10.1146/annurev.micro.55.1.255

Shi, J., Zhang, Y. B., Liu, T. K., Sun, F., & Gui, J. F. (2012). Subcellular localization and functional characterization of a fish IRF9 from crucian carp *Carassius auratus*. *Fish & shellfish immunology*, *33*(2), 258-266. doi: 10.1016/j.fsi.2012.05.014

Silvennoinen, O., Ihle, J. N., Schlessinger, J., & Levy, D. E. (1993). Interferon-induced nuclear signalling by Jak protein tyrosine kinases. *Nature*, *366*(6455), 583. doi: 10.1038/366583a0

Sironi, J. J., & Ouchi, T. (2004). STAT1-induced apoptosis is mediated by caspases 2, 3, and 7. *Journal of Biological Chemistry*, *279*(6), 4066-4074. doi: 10.1074/jbc.M307774200

Skjesol, A., Aamo, T., Hegseth, M. N., Robertsen, B., & Jørgensen, J. B. (2009). The interplay between infectious pancreatic necrosis virus (IPNV) and the IFN system: IFN signaling is inhibited by IPNV infection. *Virus research*, *143*(1), 53-60. doi: 10.1016/j.virusres.2009.03.004

Skjesol, A., Hansen, T., Shi, C. Y., Thim, H. L., &

Jørgensen, J. B. (2010). Structural and functional studies of STAT1 from Atlantic salmon (*Salmo salar*). *BMC immunology*, *11*(1), 17. doi: 10.1186/1471-2172-11-17

Skjesol, A., Skjæveland, I., Elnæs, M., Timmerhaus, G., Fredriksen, B. N., Jørgensen, S. M., ... & Jørgensen, J. B. (2011). IPNV with high and low virulence: host immune responses and viral mutations during infection. *Virology journal*, *8*(1), 396. doi: 10.1186/1743-422X-8-396

Smail, D. A., Bain, N., Bruno, D. W., King, J. A., Thompson, F., Pendrey, D. J., ... & 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. *Journal of Fish Diseases*, *29*(1), 31-41. doi: 10.1111/j.1365-2761.2005.00678.x

Song, H., Santi, N., Evensen, Ø., & Vakharia, V. N. (2005). Molecular determinants of infectious pancreatic necrosis virus virulence and cell culture adaptation. *Journal of virology*, *79*(16), 10289-10299. doi: 10.1128/JVI.79.16.10289-10299.2005

Stark, G. R. (1998). I, Kerr M, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annual Review of Biochemistry*, *67*, 227-264. doi: 10.1146/annurev.biochem.67.1.227

Stein, C., Caccamo, M., Laird, G., & Leptin, M. (2007). Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. *Genome biology*, *8*(11), R251. doi: 10.1186/gb-2007-8-11-r251

Sun, B., Robertsen, B., Wang, Z., & Liu, B. (2009). Identification of an Atlantic salmon IFN multigene cluster encoding three IFN subtypes with very different expression properties. *Developmental & Comparative Immunology*, *33*(4), 547-558. doi: 10.1016/j.dci.2008.10.001

Thomas, M., Finnegan, C. E., Rogers, K. M. A., Purcell, J. W., Trimble, A., Johnston, P. G., & Boland, M. P. (2004). STAT1: a modulator of chemotherapy-induced apoptosis. *Cancer research*, *64*(22), 8357-8364. doi: 10.1158/0008-5472.CAN-04-1864

Townsend, P. A., Scarabelli, T. M., Davidson, S. M., Knight, R. A., Latchman, D. S., & Stephanou, A. (2004). STAT-1 interacts with p53 to enhance DNA damage-induced apoptosis. *Journal of Biological Chemistry*, *279*(7), 5811-5820. doi: 10.1074/jbc.M302637200

Verrier, E. R., Langevin, C., Benmansour, A., & Boudinot, P. (2011). Early antiviral response and virus-induced genes in fish. *Developmental & Comparative Immunology*, *35*(12), 1204-1214. doi: 10.1016/j.dci.2011.03.012

Williams, K., Blake, S., Sweeney, A., Singer, J. T., & Nicholson, B. L. (1999). Multiplex reverse transcriptase PCR assay for simultaneous detection of three fish viruses. *Journal of Clinical Microbiology*, *37*(12), 4139-4141.

Wolf, K. (1988). Infectious pancreatic necrosis. *Fish viruses and fish diseases*.

Zhou, Z., Hamming, O. J., Ank, N., Paludan, S. R., Nielsen, A. L., & Hartmann, R. (2007). Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. *Journal of virology*, *81*(14), 7749-7758. doi: 10.1128/JVI.02438-06