Study of the Piscine orthoreovirus (PRV) associated with heart and skeletal muscle inflammation (HSMI) in Atlantic salmon.

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Summary

Heart and skeletal muscle inflammation (HSMI) is a challenging disease of farmed Atlantic salmon, and was first discovered in farms situated in the coastal areas of Norway. Piscine orthoreovirus (PRV) is the causative agent of HSMI but the mechanism of PRV infection in salmon is still unclear. The σNS and μNS proteins of PRV are predicted to be involved in the formation of viral factories. The aim of the present study was to investigate the co-localization of the σNS and μNS proteins in salmon cells. However, several attempts to amplify and clone the σNS and μNS genes into the bicistronic mammalian pIRES vector were unsuccessful. The PRV σNS gene was successfully amplified from cDNA and was cloned into a pCR vector for further amplification and analysis. We failed, however, to amplify the μNS gene after several attempts with different primers. The σNS gene, previously cloned into a pcDNA 3.1 vector, was therefore used and transfected into CHSE cells to identify the localisation of the σNS gene product in these cells. The results showed that the σNS protein localized both to the nucleus and to the cytoplasm of CHSE cells. In the present study we also investigated the presence of PRV and immune gene regulation in the spleen tissue of infected salmon using the experimental cohabitation challenge mode. In studies performed in freshwater (challenge study1) and seawater (challenge study 2), we quantified PRV and the regulation of immune genes using the RT-qPCR technique. In this study we determined the regulation of CD8α, granzyme, perforin 1a, perforin 1b and viral gene copy numbers in freshwater salmon injected with PRV and in their cohabitants, and also in cohabitants in the corresponding study performed in seawater. In challenge study1, we determined that CD8α was gradually increased in spleen tissue of both injected fish and cohabitants. We found that the granzyme up-regulation was associated with an increased copy number of viruses in both cohabitant salmon and virus injected salmons, and then both the viral copies and granzyme level decreased gradually in spleen. The up regulation of CD8α, perforin A1 and B1 and granzyme indicates the activation of CD8 cytotoxic T lymphocytes in Atlantic salmon in both challenge studies. These findings revealed new information about HSMI and its pathogen in Atlantic salmon.

Keywords:

Atlantic salmon, Piscine orthoreovirus (PRV), Heart and skeletal muscle inflammation (HSMI), Spleen, Immune response.

Acknowledgment

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Abbreviations and Glossary

Abbreviations

- HSMI: Heart and skeletal muscle inflammation
- PRV: Piscine orthoreovirus
- VF: Viral factories
- NS: Non-Structural
- MHC: Major Histo-compatibility Complex
- TCR: T cell Receptors
- CTL: Cytotoxic T-Lymphocytes
- VIB: Viral Inclusion Bodies
- LB: Luria-Bertani
- WPC: Week Post Challenge
- IgM: Immunoglobulin M
- ELISA: Enzyme-Linked Immuno-Sorbent Assay
- MMC: Melano-Macrophage Centers.
- CRP: C-reactive protein
- IgD: Immunoglobulin D
- μNS: Mu non structural protein
- σNS: Sigma non structural protein
- PAMP : Immune system and pathogen associated molecule pattern
- PRRs: Pattern Recognition Receptors
- TRLs: Toll like receptors
- TIR: Toll-IL-1 receptor domain
- IFNs: Interferon
- CHSE cells: Chinook Salmon Embryonic cells
- ARV: Avian ReoVirus
- GCRV: Grass Carp ReoVirus

Glossary

Alevin: A young fish

Anadromous: Fish that migrate between salt water and fresh water.

Anemia: A deficiency of hemoglobin

Apoptosis: A process of programmed cell death

Biomass: The total number of living organism in a given area.

Dendritic cells: these are antigen-presenting cells of the mammalian immune system.

Ellipsoids: A geometric surface with three coordinates axes which are circles or ellipses.

Endocytosis: A process by which cells absorb molecules by engulfing them.

Flag tag: It is a polypeptide tag that can be added to a protein by using recombinant DNA technology.

Hatching: To bring young from the egg.

Hematopoietic: A hematopoietic stem cell is a cell isolated from the blood

Hyperplasia: Increase number of cells

Lectins: The lectins are carbohydrate binding protein macromolecules that are highly specific for sugar moieties.

Lesions: Localized, abnormal structural change in the body.

Lymphoid tissue: The lymphatic system such as white blood cells, bone marrow, thymus, spleen and lymph nodes in mammals. Fish do not have lymph nedes, and the head kidney serve the role of the mammalian bone marrow.

Lysozymes: Enzymes that damage bacterial cell wall by hydrolysis

Macrophages: Large white blood cells in connective tissue or in the bloodstream that ingests foreign particles and infectious micro-organisms by phagocytosis.

Mortality: The incidence of death

Morbidity: The disease state of an individual or population

Myc tag: A polypeptide tag that can be added to a protein using recombinant DNA technology.

Myositis: Inflammation of a muscle especially a voluntary muscle.

Necrosis: A death of a circumscribed portion of animal tissue.

Net-pens: A fish rearing enclosure used in lakes and marine areas

Phagocytosis: A process in which cell engulf the foreign particles.

Phylogenic tree: The evolutionary genetic relationship between a group of organisms depicted as branches in a tree.

Roe: The mass of eggs within the ovarian membrane of the female fish

Serological: The science dealing with the immunological properties and actions of serum.

Smolt**:** Silvery salmon in the developmental stage of its first migration to the sea.

Spawn: The act of depositing eggs or sperm directly into the water typical for fish.

Teleosts: A group of bony fish, having rayed fins and a swim bladder.

Transduction: transfer of viral/ bacterial DNA from one cell to another using a bacteriophage vector.

Transfection: A process of deliberately introducing nucleic acids into cells.

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1.0. Introduction

1.1. Atlantic Salmon (*Salmo Salar*)

The scientific name of Atlantic salmon is *Salmosalar* belonging to the family *Salmonidae*. The Atlantic salmon is the most important farmed fish species in Norway. Morphologically, adult Atlantic salmon are laterally shaped and have a streamlined body. The shape and color of Atlantic salmon change with different life stages (Bostic, K. et al., 2005).

The complete Atlantic salmon breeding process begins on shore in an incubator tray. However, for wild salmon the roe is fertilized in freshwater and hatched after 60 days. When the hatching process is completed, Salmon alevins have a yolk sac on their stomach from which they draw nourishment. 10-16 months later, it has gain 60-100 gram weight and move to net pens in the sea for on-growing and maturation. Since hatching, the fish have undergone several necessary changes called smoltification and salmon at this stage are known as smolt. The salmon are kept in net pens of the sea for 14-22 months and it gain 4-6 kilogram weight. The life stages of salmon have shown in Fig.1 (Norwegian Seafood Federation and Norwegian Seafood Council., 2011).

Anadromous fish reproduce and spend their early years in fresh water. They develop through several stages before becoming a smolt, silvery salmon. Atlantic salmon first migrates to the sea for a growth phase, and later it returns to freshwater as adults. The growth opportunities are big in the highly productive aquatic environment. The greater part of anadromous fish biomass is gain in the marine environment (Flecker, A.S. et al., 2010).

The physiological, morphological and behavioral changes are the pre-requirement for Atlantic salmon to transfer from freshwater to seawater. These changes are also required for survival and growth in the marine environment and are named parr-smolt transformation or smoltification. Juvenile Atlantic salmon in freshwater has unique feature such as small mouth, pointed head, long pectoral fins, deeply forked, narrow tail stalk and sharp ended tail. Moreover, when parr migrate to seawater to become smolt fish, they become more elongated, fins become more darken, a layer of guanine crystals laid down in the skin, become more silvery in colour and obscuring the spots and marks except gills cover (Abstract of the Moscow Workshop on the Biology of Atlantic Salmon., 2004 & Atlantic salmon trust., 2014).

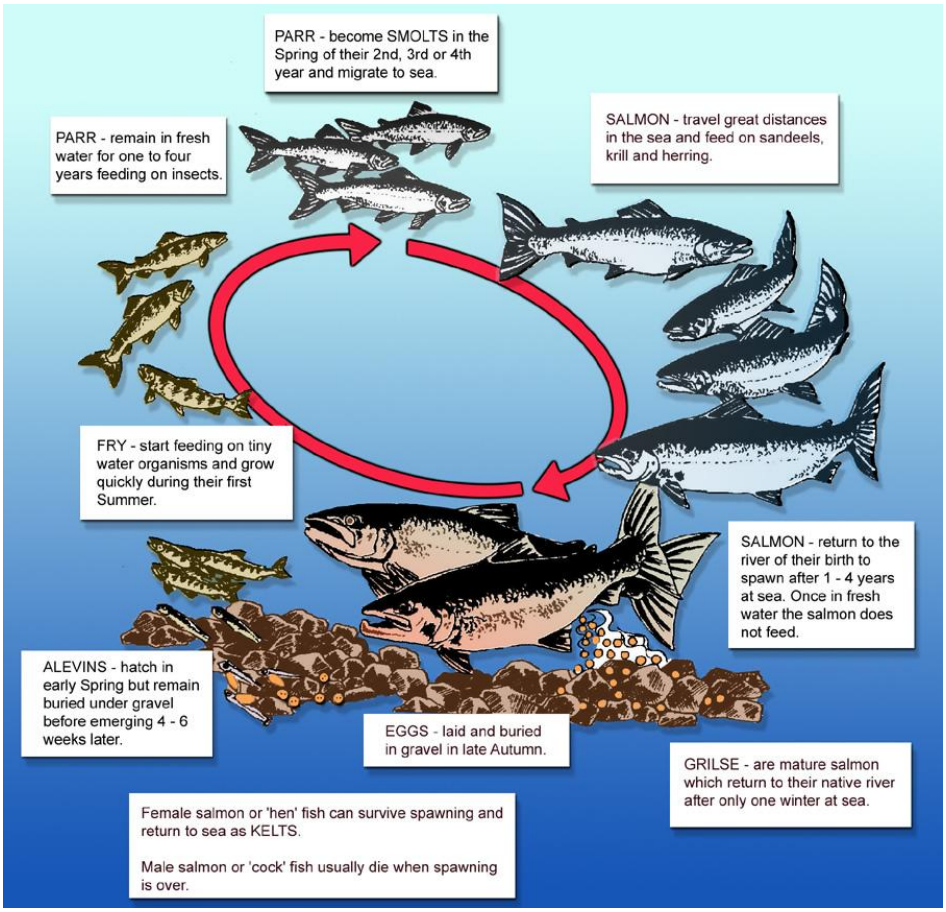

Figure.1. The life stages of Salmon
(http://www.snh.org.uk/salmonintheclassroom/salmon_lifecycle.shtml)

1.2. Aquaculture and Atlantic salmon

Aquaculture is a rapidly growing sector for food production which aims to meet the demands for animal protein in the world. The annual consumption of fish worldwide was estimated to 110 million tons in 2010, and will possibly increase to 200 million tons in 2030 (Palacios, G., 2010). Atlantic salmon fish farming holds major stakes in aquaculture and it represents more than 80 % of the total Norwegian aquaculture. About 95 % of salmon production is exported to Europe, and France is the largest export country for Norwegian salmon. Atlantic salmon fish farming has become a major industry along with the 101,000 kilometre coastline of Norway (Food and Agriculture Organization of the United Nations, 2014). Moreover, Norway has 90,000 square kilometres of aquaculture equal in size to the agricultural area of Norway, Sweden, Finland and Denmark combined. 41 diverse strains are used to produce all the Norwegian salmon. It has been reported that there are three main reasons for the acceptance of Atlantic salmon farming worldwide including versatile and adaptable nature, global health

trend and the presence of high-nutrient valued proteins. The Norwegian aquaculture industry has grown 40 % from 2000 to 2010 as shown in Fig.2 (Norwegian seafood Federation and Norwegian Seafood Council., 2011).

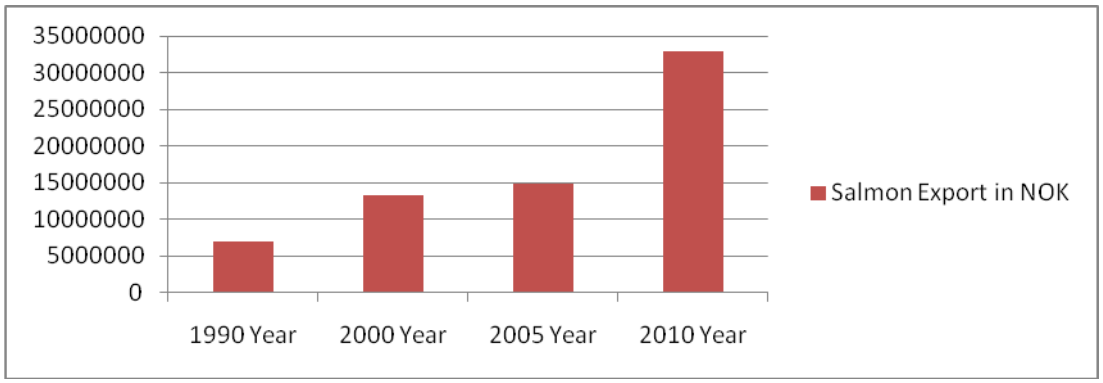

Figure.2. Salmon export to other countries of the world was plotted from the year 1990 to 2010 on x-axis and export in Norwegian kroner presented on the y-axis.

*(Norwegian Seafood Federation and Norwegian Seafood Council., 2011.*www.seafood.no)

There are several hundred salmon farms along the Norwegian coast, where the temperature and living conditions are ideal for Atlantic salmon. In the 1970, the first farms were built at the Island of Hitra (Norway). Each year, around 38 million meals of Norwegian seafood are served throughout world in around 100 different countries and eleven million are salmon meals.

1.3. Aquatic virus and viral disease

The marine environment contains millions of virus in each millilitre of water (Bergh, O. et al., 1989) and a large number of viral strains that infect marine animals can lead to death. Viral infections in commercially farmed species, such as Atlantic salmon and rainbow trout, can cause great economic losses.

The Infectious salmon anemia virus (ISAV) is a family member of *Orthomyxoviridae* and the causative agent of infectious salmon anemia (ISA) (a disease of Atlantic salmon farmed fish) (Godoy, M.G. et al., 2013). This disease was initially reported in Norway but the viral nature of the disease was only confirmed after isolation of SHK-1 salmon cell line which was

susceptible to infection. This virus has been well characterized and studied both *in vivo* and *in vitro* (Orpetveit, I. et al., 2008). ISAV has been isolated from rainbow trout in Ireland and from salmon in Chile (Kibenge, F.S.B. et al., 2001). In 2007, an ISA viral outbreak caused catastrophic economy loses to the Chilean fish industry (Godoy, M.G. et al., 2008). The experimental studies have showed that Pacific salmon are relatively resistant to the virus (Rolland, J.B. et al., 2003). The ISAV infection may not reveal clinical signs. The outbreak of ISAV in clinical carrier fish can be due to adverse environmental conditions such as stress due to rising or falling temperature and poor water quality (Lovely, J.E. et al., 1999). Normally the ISAV outbreak occurs during the spring or winter due to increase or decrease in water temperatures. The risk factors could be geographical proximity to infected marine sites, slaughterhouses, contaminated water and sharing of equipment between sites. Atlantic salmon survivors of an ISAV infection seem to be less susceptible to re-infection due to the protective immune response (Mjaaland, S. et al., 2005).The genome of ISAV consist of eight negative-sense ssRNA molecules which encodes at least 10 different proteins (Orpetveit, I. et al., 2008).

The Infectious Pancreatic Necrosis Virus (IPNV) belonging to Birnaviridae family was the first intensely studied fish virus and was the first infectious virus that was isolated in culture (Wolf, K. et al., 1960). A considerable number of IPNV strain and IPNV like viruses has been isolated from massive number of diseased and non-diseased fish species and invertebrates throughout the world (Hill, B.J. et al., 1995). IPNV was acknowledged as an acute contagious disease of salmonid of fresh water that could cause up to 100 % mortality in young salmonid. The morality rates of different disease outbreak vary because of various factors including host organisms. It is one of the most substantial diseases of salmonid aquaculture industry (Davies, K.R. et al., 2010 & Santi, N. et al., 2004 and 2005). Recently, IPNV association has been established in post-smolt and seawater stages of salmon (Christie, K.E. et al., 1988 & Roberts, R.J. et al., 2005). Aquatic birnaviruses that cause Salmonid disease have serologically resemblance with other viruses which have different characteristics such as non-virulence.

Pancreas disease was recognized in 1970 among farmed Atlantic salmon. Salmon alphavirus (SAV) is a causative agent for this disease and belong to family *Togaviridae*. A viral disease relationship between salmon pancreas disease virus and sleeping disease virus has been established. It caused outbreak in many countries such as Ireland, England, France, Germany, Spain, Italy and Norway (Hodneland, K. et al., 2005). It has been suggested that pre-exposed fish developed resistance against infection (Rolland, J.B. et al., 2003). However, subsequent

studies have shown that inactivated virus (Lopez-Doriga, M.V. et al., 2001) and recombinant, attenuated salmonid alphavirus (Moriette, C. et al., 2006) can provide good defense against pancreas disease (Fringuelli, E. et al., 2008).

Alphaviruses have an ssRNA genomes (11-12 KB in size) and spherical with a membrane envelope. The coding sequence is organized into two large, non-overlapping open reading frames (Karlsen, M. et al., 2009).

1.4. PRV and Atlantic salmon

The disease named 'Heart and skeletal muscle inflammation' (HSMI) was first identified in farmed Atlantic salmon in Norway in 1991(Kongtorp, R.T. et al., 2004) and later also reported in the United Kingdom and Chile (Ferguson, H.W. et al., 2005 & Yousaf, N.M. et al., 2012). The disease is reported in small smoltified fish (0.3 to 1kg) throughout the year, but is most frequent during spring and early summer. Atlantic salmon commonly use 5-9 months to develop the HSMI but for the challenge studies it take about 2 months. HSMI is characterized by epi-, endo-, myocardial necrosis, myositis and necrosis of the red muscle and chronic myocarditis of the epicardium and sponges, compact part of the ventricle (heart). The association of piscine orthoreovirus (PRV) with HSMI was first detected by immuno-staining of heart section from co-habitant group of salmon as shown in the Fig.3 & Fig.E4 (Finstad, O.W. et al., 2012 & Kongtorp, R.T. et al., 2004 & Kongtorp, R.T. & Taksdal, T. et al., 2004).

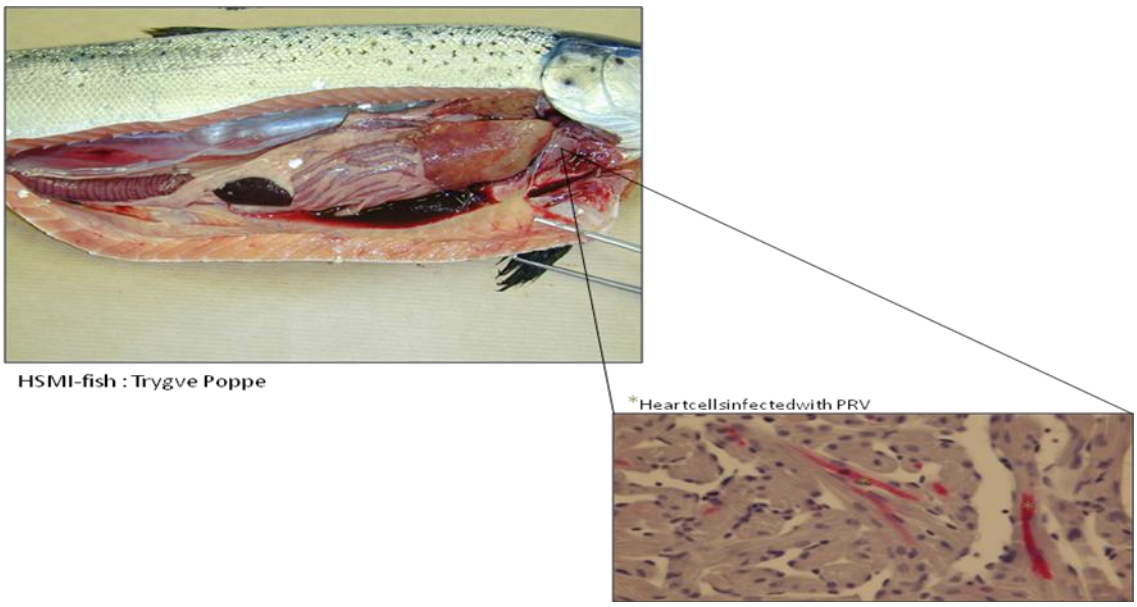

Figure.3. Salmon heart section was immuno-stained with σ-1-antibodies. The cell nucleus is colored blue (Marta Alarcon/ Øystein Finstad, Unpublished).

The disease has high morbidity as most salmon in affected sea cages showed histo-pathological lesions in the heart tissue. The cumulative mortality may reach 20 % and morbidity is near 100 % (Kongtorp, R.T. et al., 2006). Similar but not identical pathological structures in the heart have also been identified in Atlantic salmon suffering from other diseases including pancreas disease (Haugland, Ø. et al., 2011) and cardiomyopathy syndrome (Løvoll, M. et al., 2010). It has been demonstrated that HSMI can be induced by injecting tissue homogenates from HSMI diseased fish to healthy Atlantic salmon and subsequently be transmitted to cohabitating salmon (Finstad, Ø.W. et al., 2012 & Kongtorp, R.T. et al., 2006). The injected and cohabitant infected salmon develop infiltrated mononuclear cells in the epicardium with severe epicarditis and myocarditis in the spongy layer of the heart after 6 and 10 weeks respectively. Control Atlantic salmon (not injected viral infected homogenous tissue) did not show lesions (Kongtorp, R.T. et al., 2004). In 2010, the PRV sequence was published and demonstrated to be strongly correlated with HSMI (Palacios, G. et al., 2010).

1.5. Viral assembly of the Piscine Orthoreovirus

Piscine orthoreovirus (PRV) is a family member of the *Reoviridae* family. The family members of *Reoviridae* have a number of similar characteristics including a similar genome that consists of 10 segments of double-stranded RNA (dsRNA) packed in a protein particle with no membrane as described for the mammalian reovirus in Fig.E1. The best characterized orthoreoviruses are the mammalian (MRV) and the avian (ARV). Their capsids consist of inner and outer protein layers which encapsulate the genome. The inner layers cover the genome sequence and execute a key role in viral RNA synthesis. Outer layers are involved in facilitating cell attachment and viral entry into the host cell (Markussen, T. et al., 2013 & Zhang, L. et al., 2009). MRV particles come into the host cell through receptor mediated endocytosis mechanism (Tyler, K.L. et al., 1993). In this process, a large portion of the outer capsid proteins are removed from the virus by proteolysis. The inner capsid is transferred to the cytoplasm of the host cell and serves as viral core transcriptional particles (Agosto, A.M. et al., 2006). The core particles start genome transcription and form viral mRNAs. They are translated into viral proteins, and they serve as templates for the amplification of double-stranded RNA for the formation of new viruses (replication). This process of viral replication takes place in the cytoplasm (Mohan, K.V. et al., 2003 & Wei, T. et al., 2006).

The assembly of the inner capsid proteins forms core particles (progeny). These core particles are coated with outer capsid proteins and the mature virions are generated in the cytoplasm of the host cell. The specific time frame and molecular mechanism of these processes are still not completely understood. However, it has been suggested that early after viral entry, some specific viral structures grow in size and appear throughout the cytoplasm of infected cells. These cytoplasmic structures are known by various terms such as viral factories (VF), Viro-Plasms or Viro-Plasmic inclusion bodies (VIB) (Wei, T. et al., 2006 & Miller, C.L. et al., 2010). Previous studies have shown that nonstructural (NS) proteins of the *Reoviridae* family members are involved in virus replication cycles and the formation of VIB (Fan, C. et al., 2010).

Previous studies have shown that *in vitro* expression of Mammalian orthoreovirus (MRV) and avian orthoreovirus (ORV) μNS alone can form the viral factory structure. These structures were observed under light microscopes and resembled viral factories formed in infected cells (Thomas, C.P. et al. 1990). It was further shown that VIB are necessary for viral replication and RNAi-mediated knockdown of μNS expression severely inhibited viral growth (Arnold, M.M. et al., 2008). The anticipated structure of the Mammalian reovirus inner proteins, outer proteins, core particles and infectious sub-viral particles was developed and presented in in Fig.4 (Arnold, M.M. et al., 2008). It was found that the MRV μNS sequences is essential and that 250 amino acids at the C-terminus forming four distinct regions are sufficient to form the VIB in MRV (Broering, T. J. et al., 2005). These four distinct regions are predicted to form a coiled-coil domain (McCutcheon, A.M. et al., 1999). Putative zinc hooks between the coiled-coil domain function as a linker. In μNS, the deletion of 8 residues or more from the C-terminus resulted in loss of VIB formation, which demonstrates that some residues are required for formation of VIB (Broering, T. J. et al., 2005). Sequence alignments of PRV nonstructural proteins (σNS and μNS) have been constructed with MRV, ARV and GCRV as shown in the Fig.E2 & Fig.E3 respectively (Markussen, T. et al., 2013).

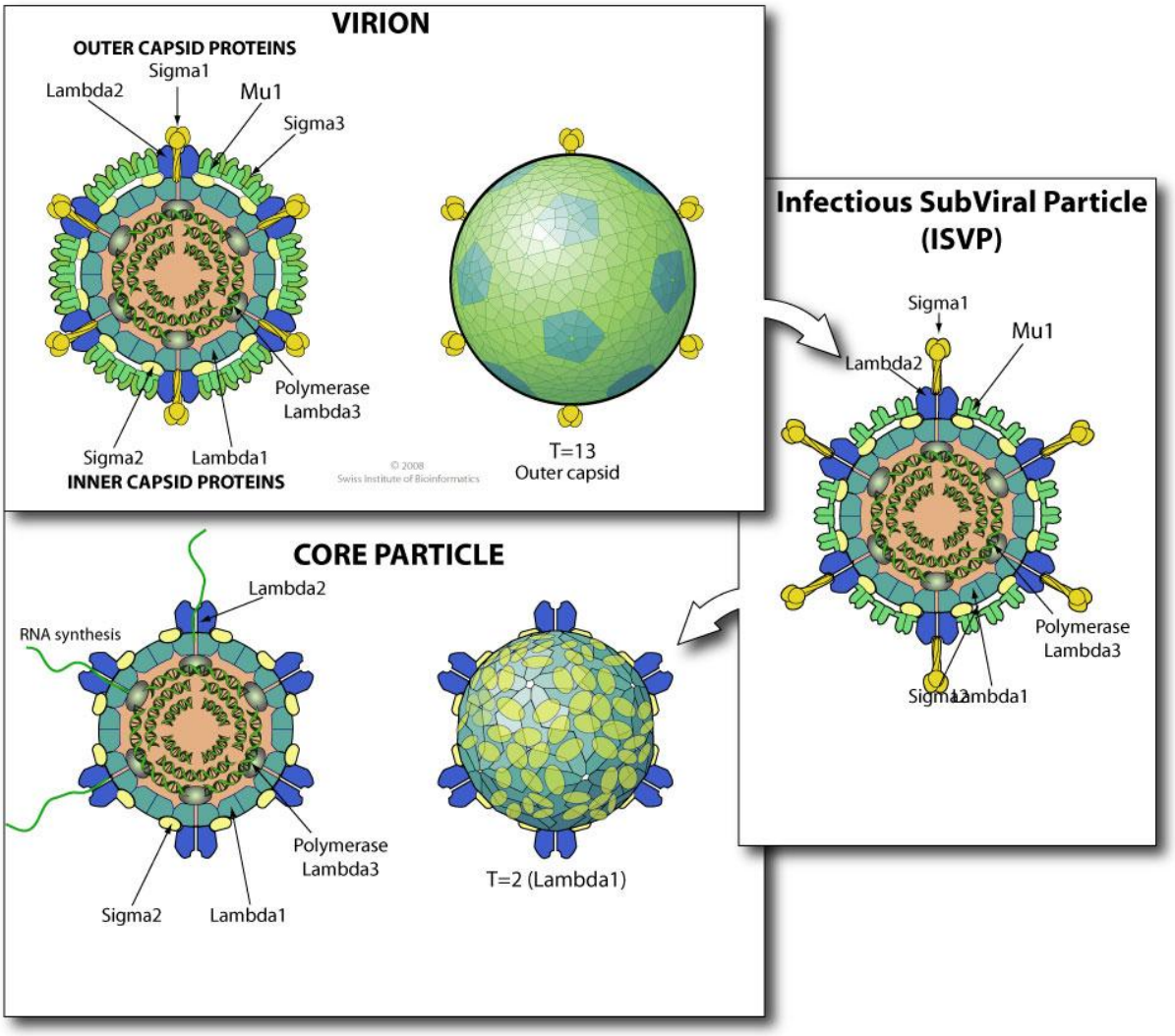

Figure.4. Diagram showed the Mammalian reovirus inner proteins, outer proteins, core particles and infectious sub-viral particles

(Swiss Institute of Bioinformatics., 2008. http://viralzone.expasy.org/all_by_species/105.html).

The PRV genome is 23320 nt long and all the segments are categorized according to length of amino acid sequence, containing three large (L1, L2, L3), three medium (M1, M2, M3) and four small (S1, S2, S3, S4) segments. Moreover, the gene segment map was made according to PRV with the other homologues segment from MRV, ARV and GCRV. The10 segments contain at least 10 (possibly 13) open reading frames, encoding the λ3, λ2, λ1, μ2, μ1, μNS and σ3, σ2, σNS and σ1 proteins. The S1, S2 and L2, segments contain small additional putative open reading frames encoding p13 (Nucleotide number: 108-482), p8 (Nucleotide number: 83-298) and p11 (Nucleotide number: 1397-1693) proteins respectively. PRV non-

structural genes (σNS & μNS) were investigated in the present study work which belongs to segment S3 and M3 respectively as shown in Fig.5 (Markussen, T. et al., 2013).

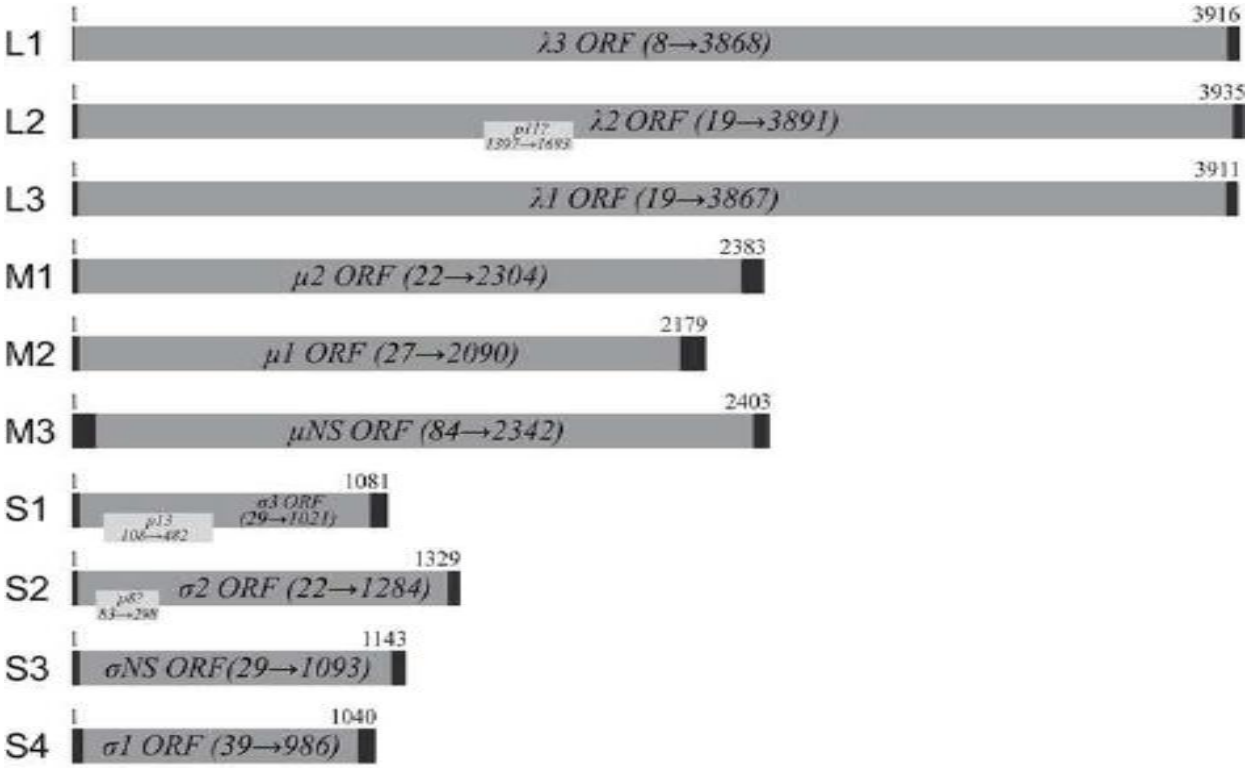

Figure.5: The PRV genome. Gene segments are assigned according to mammalian reoviruses. Open reading frames (ORFs) and putative encoded proteins are indicated by regions in grey, with start and end positions indicated. Non-translated regions (UTR's) at gene segment ends are shown in black. Gene segments L2, S1 and S2 are possibly polycistronic (Markussen,T. et al., 2013).

1.6. Innate immunity in Fish

1.6.1. Immune system morphology

The immune system organization is quite special in teleost (a group of bony fish including salmon) and has some vital differences which do not resemble the mammalians immune system. For example, fish head kidney is a vital hematopoietic organ which has functional similarities with the bone marrow in higher vertebrates. The absence of bone marrow and lymph node system is another feature which differentiates the fish and mammalian immune system. However, the main lymphoid tissues in teleost fishes are the kidney, spleen, thymus and mucosa associated lymphoid tissues associated with the skin and gills (Zapata, A. et al.,

2006 & Schroder, M.B. et al., 1998). In carp, the gut also has associated lymphoid tissues which function in eliciting immune responses, but this is not well studied in salmon (Joosten, P.H.M.K. et al., 1996).

1.6.2. Thymus

The thymus has two lobes which are represented by a thin sheet of oval lymphoid tissue (Ellis, E.A., 2001). Fish thymus is a capsule like structure that is surrounded by the lymphoid bark tissue. It is hard to distinguish clear differentiation between the cortex and medulla of the thymus among teleost. The initial development of the thymus varies between teleost species, according to the effect of temperature on growth and the thymus is responsible for the production of T cells. The structural components of teleost are elaborated in Fig.6 (Tort, L. et al., 2003).

1.6.3. Head Kidney

In teleost fish, the Head kidney performs the same function as bone marrow in vertebrates. It works as the largest site of haematopoiesis until adulthood of teleost fish (Zapata, A. et al., 2006). The head kidney is well-developed after hatching in salmonids and its main function is to produce red blood cells and granulocytes. A major portion of antibodies are synthesized in the head kidney. Macrophages may aggregate into structures called Melano macrophage Centers which bind to the foreign particles (Press, C.M. et al., 1994). The macrophages work as a bridge between the innate and adaptive immune response (Ellis, A.E., 2001 & Ferguson, H.W. et al., 2005).

1.6.4. Spleen

The spleen is composed of lymphoid tissue and may be divided into a red and white pulp, although the white pulp is poorly developed in teleosts. The ellipsoids are thick wall capillaries and clustered together around the lymphoid tissue. Cells (macrophages) along the walls are dynamically participating in the phagocytosis of antigens. Moreover, ellipsoids take

part in plasma filtration and trapping of substance from the blood stream (Ellis, A.E., 2001 & Espenes, A. et al., 1995).

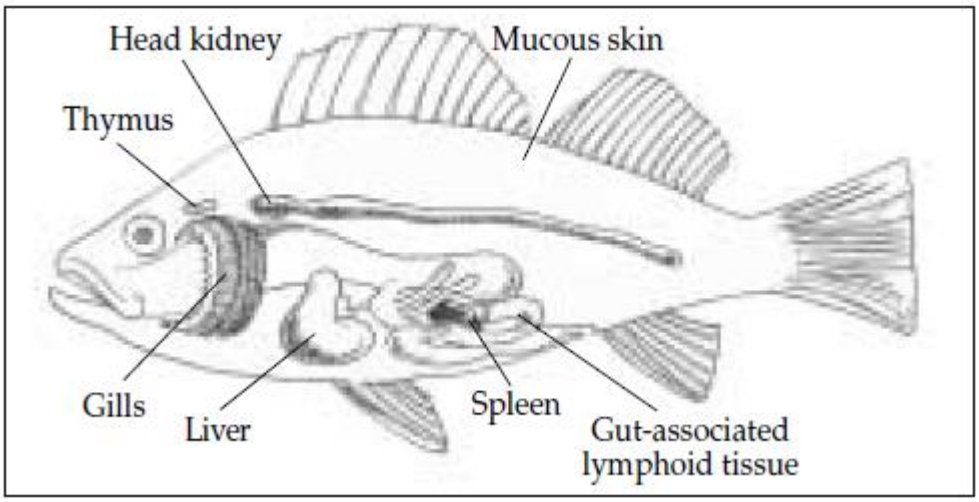

Figure.6: Immune structures in teleost fish (Tort, L. et al., 2003).

1.7. Immune system component

1.7.1. Innate Immunity in teleost

The initial response is a fundamental component of immunity to combat the pathogens due to the presence of the inadequate adaptive immune system, time needed for proliferation, maturation and memory of the adaptive lymphocytes (Whyte, S.K. et al., 2007). It may be divided into the epithelial/ mucosal barrier, circulating antimicrobial molecules and cellular components.

1.7.2. Physical Barriers in teleost

The first physical barrier to prevent the infection, is the flakes, the mucus surface of skin, gills and the epidermis of teleost (Shepard, K.L., 1994 & Ellis, A.E., 2001).The composition of mucus includes lectins, lysozymes, pentraxins, antibacterial peptides, complement proteins and immunoglobulin M which prevent entry of pathogens (Rombout, J.H. et al., 1993 & Boshra, H. et al., 2006 & Saurabh, S. et al., 2008).The antimicrobial polypeptide secretions were found in mucus, liver and gill tissue of teleost fish (Hellio, C. et al., 2002 & Birkemo, G.A. et al., 2003). The polypeptides play vital role in defence mechanism against virus and

bacteria and have the ability to break down bacterial walls. In addition, epidermis prevents the entry of foreign agents by cellular hyperplasia, mucus secretion and thickening (Ellis, A.E., 2001& Chinchar, V.G. et al., 2004 & Maier, V.H. et al., 2008).

1.7.3. The immune system and pathogen associated molecule pattern (PAMP)

The pathogen associated molecule patterns (PAMPs) are the highly conserved molecular motifs displayed on the surface of most pathogen. The immune system recognized these motifs with the help of Pattern Recognition Receptors (PRRs) (Elward, K. et al., 2003 & Werling, D. et al., 2003). A major class of receptors that recognize molecular patterns of pathogens such as bacteria, fungi, protozoa and viruses are Toll-like receptors (TLRs). These receptors are identified on various types of immune cells such as macrophages, dendritic cells, B cells, specific T cells and some non-immune cells. TLRs start intracellular signal transduction upon recognition of pathogens. Intracellular signal transduction induces the expression of genes which are involved in inflammation, antiviral responses and antigen presentation (Akira, S. et al., 2006 & Kawai, T. et al., 2005). 11 TLRs have been recognized in mammalians and several more are identified in fish. TLRs agonists are either live pathogens or isolated PAMPs that work as immune-stimulants in activation of the immune system (Kawai, T. et al., 2005 & Meylan, E. et al., 2006 & Meijer, A.H. et al., 2004 & Jault, C. et al., 2004).

1.8. Cellular components of teleost

Phagocytosis is the most important mechanism involved in the early immune defence of teleost. The neutrophils and macrophages are main phagocytic cells, responsible for the execution and elimination of bacteria and other pathogens (Fischer, U. et al., 2006). In fish, also other immune cells have been found to be phagocytic, including some B cells. The cytotoxic cells in the innate immune system are known as natural killer cells and are responsible for early execution mechanism in mammals. Fish cytotoxic cells also have the capability to eliminate the parasites because these have similar characteristics as mammalian cells. The natural killer cells can induce apoptosis in infected cells. In catfish, cytotoxic cells were found in anterior kidney and spleen in addition to the blood circulation (Shen, L. et al., 2002).

1.9. Adaptive Immunity in teleost

Teleosts produce several antibodies, and have monomer and tetramer IgM in their serum. The expression factors behind monomer IgM in serum are not identified yet and IgD, the second immunoglobulins isotype was found in fish (Wilson, M. et al., 1997). The concentration of IgM in Atlantic salmon serum is quite low compared with the other teleost such as Japanese eel (Uchida, D. et al., 2000). In teleost species, antibodies are found in the skin, gills, gill mucus, bile, intestine and plasma. Moreover, specific antibodies can be produced in skin and in the intestine without systemic stimuli, and mainly consist of another type of antibody, igT (Rombout, J.W. et al., 1986 & Cain, K.D. et al., 2000 & Hattern, F. et al., 2001 & Uribe, C. et al., 2011).

Teleost develop memory response after initial exposure to the antigen but upon secondary exposure the T lymphocytes respond in both antigen-dependent and independent manners. The concentrations of antigen-specific B cells in the spleen are directly proportional to the frequency of B-cell specific antigen precursors (Arkoosh, M.R. et al., 1991). In Atlantic salmon, T-cell mediated immunity is still not very thoroughly studied. T cells are involved in humoral (antibody-mediated) and cell mediated (cytotoxic) immunity. The different functions of T-cells are linked to the surface expression of CD4 and CD8 receptors, and these receptors are involved in the interaction between the T-cells receptor complex and the antigen-presenting major histocompatibility complex (MHC). Both receptors consist of alpha and beta chain, and each chain participates equally in recognition of MHC.Intracellular foreign or endogenous antigens, presented by virus infected cells and a tumour cell in MHC type I (MHCI) activates the T-cells which have T cell receptors (TCR) and CD8 receptors on their surface. The CD8 receptor molecule is comprised of either two alpha chains or one alpha and one beta chain. Intraepithelial lymphocytes and natural killer cells primarily express the homo-dimeric CD8 molecules.When naive CD8+ cells recognize MHCI-presented peptides, they proliferate and differentiate into cytotoxic T-lymphocytes (CTL).CTLs contain cytotoxic granules in their cytoplasm, and adhere firmly to specific targets on infected cells and initiate cell killing by delivering granule proteins (granzymes) into the target cell. Granzymes are transported by receptor-mediated endocytosis with the help of Perforin and sulfated glycoproteins called serglycin. Granzyme activate caspases which are present in the cytoplasm of infected cells, and these caspases further activates the mechanism of apoptosis.

1.10 CD8α and CD8β receptors of immune system

The CD8α and CD8β are transmembrane glycoprotein which is present on the surface of the cytotoxic T cells and natural killer cells. They serve as co-receptor for the T cell receptors. CD8 receptors bind to the major histocompatibility complex (MHC) class 1 protein molecules that are present on infected cells (APCs) (Gao, G.F. et al., 2000). The CD8 receptor is composed of CD8α homo dimers and CD8αβ hetero dimers and consists of a pair of CD8 chains. CD8β is required for surface expression of CD8α in cytotoxic T cells. These glycoproteins are members of the immunoglobulin super family and the molecular weight of CD8α is 34 kDa (Bangs Laboratories., 2013). The immunoglobulin variable like domain is attached to the membrane by a thin stem. The extracellular domain of CD8α interacts with the Class 1 MHC molecule of APCs and this adhesion ties both cells together during the activation of specific cytotoxicity (Devine, L. et al., 1999).

1.11 Granzyme and perforin enzyme of immune system

Granzyme are serine proteases and these enzymes cleave protein ku70 that is a key double strand break repair protein in target cells (Zhu, P. et al., 2006). The granzyme are released through cytoplasmic granules within cytotoxic T cells and natural killer cells (Bots, M. et al., 2006). Granzyme are also present in the rough endoplasmic reticulum, golgi complex and the trans-golgi reticulum of the cell (Peters, P.J. et al., 1991). These enzymes have the capability to induce the mechanism of apoptosis within virus infected cells.

Perforin cytolytic protein has been found in the granules of CTLs and natural killer cells. These proteins penetrate the membrane of viral infected/damaged cells through the mechanism of degranulation. Degranulation is a cellular process that release antimicrobial and/or cytotoxic molecules to destroy the invading microorganisms or damaged/infected cell. Perforin inserts itself into the plasma membrane of the target cells and form pores. It has been studied that the perforin and granzyme can be released in membrane enveloped vesicles upon CTL-TC interaction (Peters, P.J. et al., 1991). These pores work as a gateway for granzyme to enter the damaged/ infected cell and activate apoptosis.

2.0 Aim of Study

In the present study we have investigated the localization of the non-structural proteins σNS in CHSE (Chinook Salmon Embryo) cells by immune fluorescent staining. These non-structural proteins are considered to take part in the construction of the viral factories in the host cells. We have also analysed two Atlantic salmon challenge studies for the gene expression of cytotoxic cell markers during the course of HSMI.

3.0 Material and Methods

3.1. Plasmid vectors used in this study

The pIRES vector is a bicistronic vector used to determine the co-localization of proteins and have two multiple cloning site (MCS)-A and MCS-B. The vector can be used to monitor the localization of the cloned gene product by using a fluorescent tag. Two genes of interest can be cloned into the same vector to determine co-localization of the protein products. Moreover, it can be used to monitor protein modification (Genecopoeia, 2014).

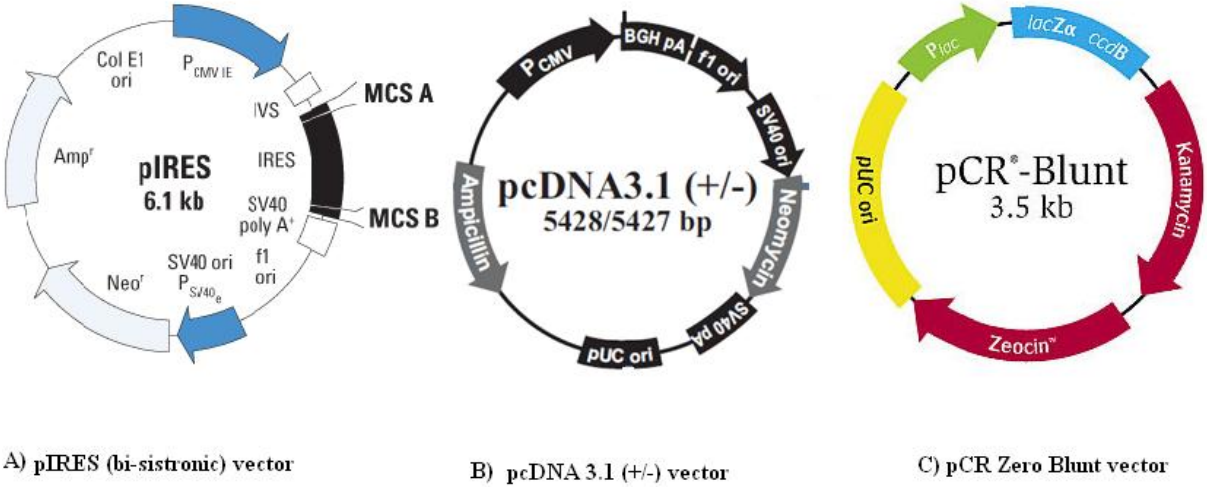

Figure.7: Diagram (A) show the structure of pIRES (bicistronic) vector with the size 6.1 KB (Clonetech, 2014). Diagram (B) show pcDNA 3.1 (+/-) vector with size 5.4 KB (Lifetechnology, 2014). Diagram (C) show pCR-Zero Blunt vector with size 3.5 KB (Lifetechnology, 2014).

The pcDNA 3.1(+/-) is a 5.4 kb vector designed for the high transient expression of cloned genes in mammalian cells. It contain cytomegalovirus immediate early (CMV) promoter for high level expression in mammalian cells, and a multiple cloning site in the forward and reverse orientations to facilitate cloning. This vector has a neomycin resistance gene. The pCR Zero Blunt vector was used for direct insertion of blunt ended PCR product. The vector contains the *ccdB* gene for positive selection and only those clone which have insert grow. T7 forward and M13 reverse primer sites are flanking the insertion site in the vector, and can therefore be used to verify sequence composition of the cloned insert (gene). The pCR vector

has MCS, kanamycin & ampicillin resistance genes. Map of all three vectors are showed in Fig.7.

3.2. Bioinformatics tool and techniques

CLC Bio software was used for in-Silico Cloning Workflow. This software was also used to design primers for amplification and sequencing of the µNS and σNS gene. The forward primers for amplification of the σNS and µNS gens were designed to contain the myc tag and Flag tags sequences respectively as shown in Table.5. These tags were used for the detection of the protein *in vivo* during microscopic examination. The protein sequence and molecular weight of these tags are shown in the Table.3.

3.3. Propagation and isolation of pIRES vector

The pIRES vector was transformed into the DH5**α™** (Invitrogen) competent cells to propagate the vector and to obtain a sufficient amount of the vector for the cloning work. 176 ng pIRES vector was transferred into pre-marked competent cells tube. It was incubated for 5 minutes and then heated at 42 °C for 30sec in a water bath to transform the pIRES vector into DH5**α ™** competent cells. The sample was placed on ice for 2 minutes and then a volume of 80 µL SOC medium then incubated at 37 °C for 1-2 hours at 250 rpm in shaking incubator. A specific volume of cells was spread over two pre-marked agar plate with the help of a sterile stick. This petridis contained LB medium containing ampicillin (50µg/ml). Ampicillin resistance colonies were cultured for 4-5 hours in 8 ml LB media containing an ampicillin 50 µg/ml. The culture was transferred to a 1000 ml flask containing 600 ml of LB media with ampicillin concentration 50µg/ml. This culture was incubated at 37 °C overnight. The bacteria cells were centrifuged at 4 °C and 5000 g for 15 minutes. Plasmids were isolated according to the NucleoBondXtra kit. The bacterial cell pellet was lysed and neutralized to precipitate the protein, chromosomal DNA and other cellular debris. A filter column was equilibrated and the lysate was added, washed and eluted using the kit buffers. The plasmid DNA was then precipitated using isopropanol and centrifuged at 12000x G/4°C for 30 minutes. The pellet was washed with 70 % ethanol centrifuged and after removing the ethanol the pellet was air dried for 10 minutes, and finally dissolved in endotoxin free H_2O and stored at -20 °C.

3.4. Polymerase Chain Reaction for σNS and μNS

TheTotal RNA from HSMI diseased Atlantic salmon was used as template to synthesize the cDNA as described in material and method section 3.11 which then was used as template to PCR amplify the σNS and μNS genes. The primers for PCR amplification are shown in Table.2. The reaction mixture to run a standard PCR was comprised on 30 µL PCR Grade water, 5 µL (10X) PCR Buffer for KOD Hot Start DNA Polymerase, 5 µL dNTPs (final concentration 02mM), 2 µL $MgSO_4$(final concentration 1mM), 1 µL DNA template, 3 µL of 5´ primer (5mol/ µL, final concentration 0.3uM), 3 µL of 3´ primer (5mol/ µL, final concentration 0.3uM) and 1 µL of KOD Hot Start DNA Polymerase (1U/µL). The reaction mixture was mixed and centrifuged briefly. The PCR was run by using the following cycle parameters: 15 minutes at 95°C to activate the polymerase, denature 15 sec at 95 °C, anneal 30sec at 54 °C, and extend for 30 sec at 72 °C and this process was repeated for 30-40 cycles. The PCR kit of Qiagen was used to clean the PCR product according to manufacturer instructions.

3.5. Plasmid Construct and touch down PCR

A database name 'CLC work bench' (CLC bio) was used to *in silico* construct a recombinant plasmid vector and a Clontech online database was used to design the primers for infusion cloning (Clonetech, 2014).The infusion primers for insert amplification were added 15 extra bases on 5 end; these 15 bases are homologous at one end of the DNA fragment that will join with the pIRES plasmid vector. A touch-down PCR technique was used to produce a series of cycles with progressively lower annealing temperature. The annealing temperature range was extended up to 15 °C from a few degrees above the estimated melting temperature to 10 °C or so beneath. A program was set in which annealing temperature was decreased 1 °C after 2-3 cycles per degree from 62 °C to 50 °C that it was followed by 15 additional cycles at 50 °C. The reaction mixture for PCR was composed of 30 µL PCR Grade water, 5 µL (10X) PCR Buffer for KOD Hot Start DNA Polymerase, 5 µL dNTPs (final concentration 02mM), 2 µL $MgSO_4$ (final concentration 1mM), 1 µL DNA template, 3 µL of 5´ primer (5mol/µL, final concentration 0.3uM), 3 µL of 3´ primer (5mol/µL, final concentration 0.3uM) and 1 µL of KOD Hot Start DNA Polymerase (1U/µL). The reaction mixture was mixed and centrifuged briefly. PCR reaction was run with the following cycle parameters: 2 minutes at 94 °C to activate the polymerase, denature 15 sec at 94 °C, anneal 30sec at 62 °C, extend for 45 sec at

74 °C and this process was repeated for 2-3 cycles per degree from 62 °C to 51 °C annealing temperature. However, at 50 °C this process was repeated for 15 cycles. Normal amplification of PCR product was determined on a bio-analyzer by using the Agilent DNA 7500 kit and this PCR product was stored at -20 °C.

3.6. Purification and detection of PCR product

The amplification of PCR product was tested on Agilent DNA 7500 kit with the help of a bio-analyzer. DNA gel matrix and DNA dye concentrate were equilibrated at room temperate for 30 minutes. A volume of 5 µL of the ladder was pipette in all 12 samples well and the ladder well.1 µL of DNA ladder was pipette in the mark ladder well. A volume of 1 µL of amplified PCR product was pipette and rest of empty wells were filled with deionized water. This chip was vortex at 2400 rpm for 1 minute and run in the Agilent 2100 bio-analyzer within 5 minutes according to standard protocol of kit. A vector pIRES was linearized with the help of EcoR1 enzyme. The reaction mixture was comprised on following reagents; 2 µL 10X restriction buffer (Buffer H), 10 µL DNA template (940 ng) (pIRES Maxi), 2 µL Enzyme I (EcoR1) and 6 µL RNase free water. The reaction was incubated for 2 hours on a water bath and then resulting linearize vector was stored at -20°C. The linearize vector was run on 1 % agarose gel to get pure linearize vectors. A volume of 10 µL of sample solution (linearize vector) was mixed with 2 µL DNA loading buffer before loading it in a well of 1 % agarose gel and 10 µL of 1KB ladder was used as a standard weight marker. The agarose get was run at 100 V for 1 hour. It was transferred on a ChemiDoc™ XRS + imaging system to get the picture of a DNA gel. Later on, it was transferred on the High Performance Ultraviolet Trans-illuminator to visualize and cut the DNA band of interest with the help of a blade. The DNA isolation kit was used to extract the DNA from the agarose according to described protocols. The quantity of DNA concentration was measured using Nanodrop instrumentation and the sample stored at -20 °C.

3.7. Cloning and Transformation

An efficient infusion cloning was achieved with the help of defined concentration of following reagents; 3µL Purified PCR fragment 10-200 ng, 1µL linearized vector 50-200 ng, 2 µL 5X infusion HD Enzyme Premix and 6µL Deionized water. Form a positive control 2µL

of 2kb control insert, 1µL of pUC19 control vector, 2µL 5X infusion HD Enzyme Premix and 5 µL deionized water was taken in an Eppendorf tube. However, in a negative control, all regents of positive control were used instead of control insert. This master mix was mixed well and it was incubated for 15 min at 50 °C then it was placed on the ice. This cloning reaction was stored at -20 °C. The procedure of the pIRES vector transforming was described above in section 2.2.

3.8. Sequencing

DNA sequencing involves in making copies of the original DNA and it is considered a key step to determine the sequence of cloned gene. Sanger sequencing reaction uses nucleotides as building block of DNA and DNA polymerase enzyme to add new bases to a growing DNA chain. The primers serve as starting point for building a new DNA chain and bind to a specific DNA sequence at the DNA molecule and start adding nucleotide in growing chain of DNA. The DNA polymerase enzyme is responsible to facilitate the sequencing reaction and growth of DNA chain. Plasmids were sequenced using BigDye Terminator v3.1 cycle sequencing kit on an automated ABI Prism 3130 Genetic Analyzer (Applied Biosystems, CA, USA). The primers for sequencing were the T7F and M13R primers (Table.7). Prior to capillary electrophoresis, extensions products were purified using ethanol precipitation and the purified products were dissolved in ultrapure formamide (Applied Biosystems). The sequencing data was analysed with the help of the CLC Bio Main workbench (Qiagen).

3.9. Transfection

3.9.1. Preparation of CHSE cells

Media (L15 / 10 % serum / Gentamicin) was removed from the CHSE cell flask. The adherent cells were washed with 10 ml PBS. TrypLE express (2ml, life technologies) was added to detach the CHSE cells. The flask was kept at RT for 15 min and hit several times to detach the cells from the bottom. In this flask 10 ml cell media was added to resuspend the cells and rinse / transfer to a 50 ml tube. A volume of about 10 µl was taken to count the number of viable cells and the rest was centrifuged for 5 minutes at 1000 rpm to pellet the cells. Cells

were then resuspended to 1 mill / ml in fresh media L15 /10 % serum / Gentamicin in a new flask.

3.9.2. Cell counting chamber slide and viability examination

The number of cells was counted with 'Countess Automated Cell counter' (Invitrogen Life Technologies). 10 ul volume was taken from the cell media. It was mixed with 0.4 % trypan blue stain in a 1:1 ratio and pipetted into a cell counting chamber slide (Invitrogen). On the plate, cells were added with 0,3 mill / well in a 24-well plate (20 µl) togather with 750 µl media and left for 24 h. The Plasmid mix was prepared by adding 4 µg of expression Plasmid in a 120µl volume of optiMEM and it was mixed in an eppendorff tube for each transfection (4 wells).The Xtremegene master mix was prepared by adding 8 µl Xtremegene transfection reagent (Roche) in 120 µl volume of OptiMEM for each transfection. It was estimated that for 4 transfections (8 x 4) 32 µl Xtremegene transfection reagent and (120 x 4) 480 µl OptiMEM was needed. A volume of 120 µl Xtremegene master mix was added into each plasmid mix and was mixed by pipetting. It was placed at room temperature for 15 min. A volume of about 50 µl of the plasmid/Xtremegene mix was added per well of cells.

3.9.3. Immunofluorescence staining

Immunofluorescence technique was used in the present research work to detect the proteins expressed in the transfected cells. The specific primary antibodies bind to their antigen and secondary antibodies conjugated with fluorescent dyes are added to target fluorescent dyes to the protein within the cell. This technique enables us to visualize the distribution of the target molecule in the cell. We use DAPI to label the nucleus (binds to DNA). The primary antibody serum used to target σ1 and σ3 was prepared in rabbits at the Norwegian veterinary School. The primary mouse antibody targeting the Flag tag of σNS was from Sigma Aldrich. Secondary antibodies targeting mouse and rabbit IgG labelled by Alexa 488 or Alexa 595 dyes were from LifeTechnologies.

3.10. Fish experiment and sampling

In the first challenge study (V2971 FW) salmon parr with an initial average weight of 20 g were used. The inoculums were homogenized tissue (heart, spleen and head-kidney) from fish

from a field outbreak of HSMI and contained a high load of PRV as determined by RT-qPCR. The inoculum was confirmed free of other commonly known salmon pathogens. 50 % of the fish (shedder group) were anesthetized, given 0.1 mL inoculum by intraperitoneal (i.p.) injection, marked by shortening the outer left maxilla and put in a tank containing the same amount of untreated fish (cohabitants). Six fish of the cohabitant group were sampled every second week starting at 6 weeks post challenge (WPC) ending at 12 WPC. In addition, three shedder fish were sampled. Control samples from 6 fish were collected before initiation of the experiment. In the second experiment, (V3007) smoltified salmon with an average weight of 80g were used in the same cohabitation model. Only cohabitant fish were analyzed in this study, and six fish were sampled weekly from WPC 4 to WPC 8. Spleen samples were collected on RNA later, and stored at -20 °C.

3.11. RNA Isolation, RNA quality analysis and cDNA synthesis

A small tissue sample (spleen, 10-20 mg in weight) of an infected Atlantic salmon was transferred to an Eppendorf tube. One stainless steel bead (0.15mm) and a volume of 500 µL Qiazol reagent (Qiagene) was added to the sample. The tissue was disrupted and homogenized by using the TissueLyser II at 25 Hz frequency twice for 5 minutes and then the sample was incubated at room temperature (RT) for 5 minutes. 200 µl chloroform was added the sample and vigorously shaken by hand for 15 seconds. After incubation at RT 2-3 minutes the sample was centrifuged at 12000g for 15 minutes at 4 °C. The aqueous phase 350 µL was transferred into a new Eppendorf tube and 350 µL of 70 % ethanol was added. The complete sample was transferred into an RNeasy spin column. Bound RNA was washed once with 700 µL buffer RW1 (RNeasy kit) and twice with buffer RPE (RNeasy kit) according to the manufactures instructions. To completely dry bound RNA, a final centrifugation step at 13000 rmp for 1 min was performed before RNA was eluted in 30 µL RNase free water. Eluted RNA was stored at -80 °C. The random samples of RNA were selected form each time span for testing the RNA quality with the help of Bio-analyzer (Agilent technologies). Standard kit protocols were followed to perform the quality analysis of RNA and the regents to produce cDNA are presented in Table.6.

10 µL RNA (50 ng/µl) was prepared in a small PCR tube and heated at 65 °C for 5 min to denature viral dsRNA and then immediately placed once for 5 minutes. Contaminating genomic DNA was eliminated from the sample by adding of 2 µL 7X gDNA and 2 µL RNase

free water to RNA sample. This 14 µL reaction mixture was incubated at 42°C for 2 min and then placed immediately on ice. A reverse transcription master mix was prepared by pre-mixing 1µL Quantiscript Reverse Transcriptase, 4 µL Quantiscript RT buffer (final concentration 1X), 1 µL RT primers per sample, and then transferred to previously prepare 14 µL reaction mixture and giving a final reaction volume of 20 µL. This reaction was incubated for 30 minutes at 42°C, followed by 3 minutes at 95°C to inactivate the Quantiscript Reverse Transcriptase enzyme. The cDNA samples were stored at -20°C until used.

A standard curve was created at the cDNA synthesis level for the qPCR analysis. To prepare the standard curve, 12 random samples representing the different time points (from week 0 to week 12) were selected and 2ul RNAs (50 ng/µL) was taken from each sample and mixed in a small PCR-tube. A PCR strip was marked with different RNA concentrations. The cDNA was prepared as for individual samples.

3.12. Quantitative Polymerase Chain Reaction (qPCR)

Quantitative RT-PCR (qPCR) assay was used to determine the transcript levels of specific cytotoxic cell markers including viral load, elongation factor, CD8α, granzyme, perforin 1a and perforin 1b. In this study, SYBR green was used to monitor dsDNA synthesis, the reference gene elongation factor (EF)-1-α was used for sample normalization and the ROX reference dye was used as assay control. Each sample was analysed separately in reactions containing 5 µL 2x SYBR Green PCR master mix (Thermo Scientific), 0.5 µL 10 µM forward and reverse primer and 5 µL cDNA in a final volume of 900 µL.

The qPCR assay was analysed on Stratagene Mx3005P instrument using following thermal profile: 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, 58°C for 30 sec and 72°C for 30 sec. Amplicon dissociation curves were recorded after 40 cycles from 58°C to 95 °C. A file setup of a 96-well assay plate was prepared in which the first two columns of the plate contain samples for standard curve preparation. The standard curve concentration ranged from 40 - 6.25 ng and the last sample of standard curve was negative control. The remaining of the qPCR plate comprised experimental samples analysed in duplicates. The data were analysed using the MxPro-Mx3005P software. Average of duplicate quantitative copy numbers for SYBR Green and ROX dyes were summed separately for each sample. The sample value of SYBR Green was divided by the value of ROX dye for each sample. All the samples which

belong to same time point (week number) were summed up again to get average number. The standard deviation curve was made by the using average sample values for sample. Moreover, the primer sequence of immune genes (CD8α, EF1α, PRV-S1, Perforin 1a, perforin 1b and Granzyme A) and PRV σ3 are shown in the Table.4.

4.0. Results

4.1 Cloning of PRV NS proteins

4.1.1 Amplification of the PRV genes σNS and μNS for cloning

Spleen samples from an outbreak of HSMI in 2007 (Marine Harvest 050607) was used to PCR amplify the PRV genes as describe in 3.10. A PCR product of the expected size of the σNS gene was amplified (Fig.A1 lane: 2 & 3). The putative σNS PCR product was purified and cloned into the pCR-Blunt vector for sequence verification (Appendix 9). Several attempts to amplify the μNS gene failed (Fig. A1 lane: 4 & 5). Several primers sets and PCR conditions were tested without result (data not shown).

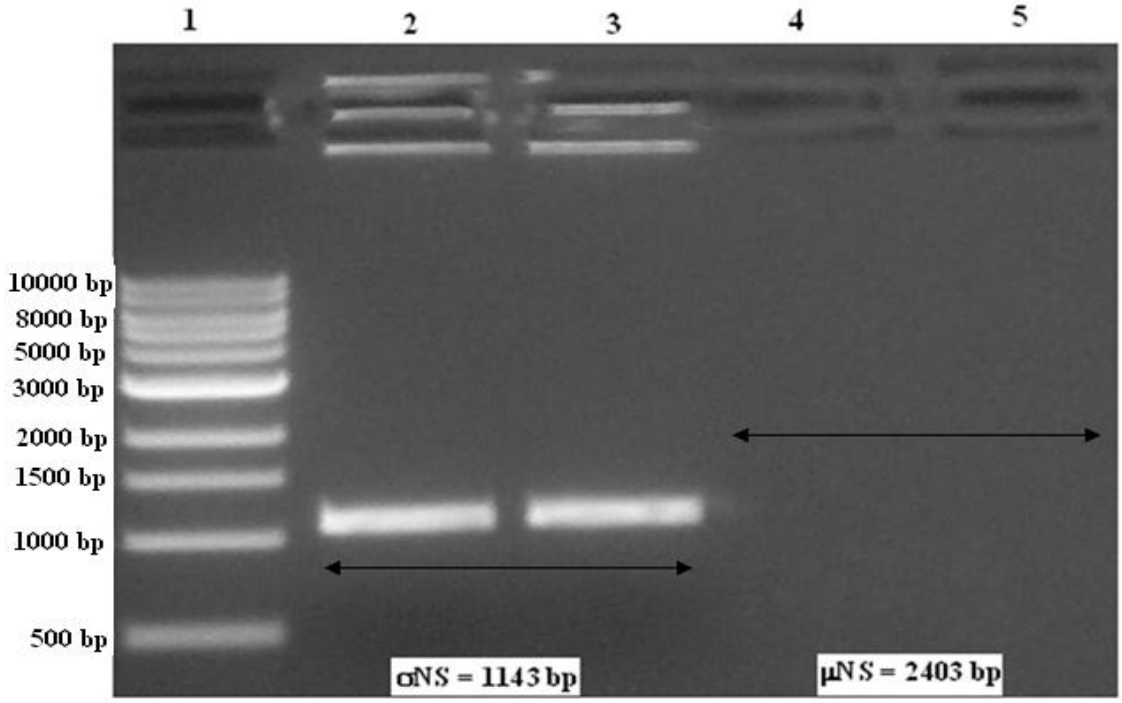

Figure.A1. 1 % agrose gel was used to determine the amplified product of the σNS and μNS genes. The PRV gene σNS is shown as the expected bands of 1143bp but no band for μNS gene was determined on the gel. Lane1: 1kb ladder, 2,3: σNS, 4,5: μNS.

4.1.2. Enzyme digestion analysis of PRV gene σNS-pCR clone and pIRES vector

Plasmids from three putative positive pCR-σNS *E.coli* clones were analysed by restriction digest analysis to select clones with inserts. Plasmids were digested with EcoR1 Fig.A2 shows the cloned PCR product with expected band size of 1143bp and pCR VECTOR 3.5 KB band size on the 1 % agarose gel as shown in the Fig.A2. These positive clones were sequenced as described in the material and method section 2.8. One positive clone which contained the full σNS gene with Myc tag at N-terminal in pCR vector was identified as shown in Figures.E6 & E7.

Sequence alignment of the cloned σNS were aligned with a previously cloned σNS gene (Markussen, T., 2013) and revealed a few silent mutations as shown in the Fig. E6. The σNS gene were attempted to clone into the pIRES vector without success. It was expected that the concentrations or reagent of reaction mixture were not optimal that's why we failed to clone σNS into pIRES vector.

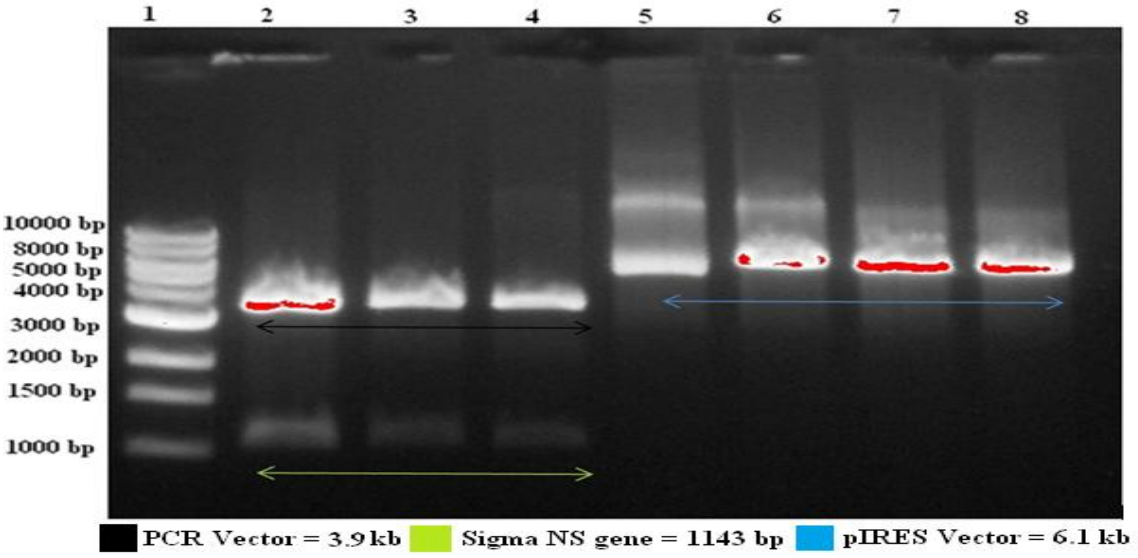

Figure. A2. Agarose gel (1 %) enzyme digestion analysis of σNS-pCR clone and pIRES vector (6.1kb). Lane1: 1kb ladder, 2, 3, 4: σNS-pCR vector clones plasmid, NEB3 buffer and EcoR1 enzyme, 5: Negative control for pIRES plasmid, NEB3 buffer, 6: pIRES plasmid, NEB3 buffer and Not1 enzyme, 7: pIRES plasmid, NEB3 buffer and Sal1 enzyme, 8: pIRES plasmid, NEB3 buffer and Not1 enzyme, 7: pIRES plasmid, NEB3 buffer Sal1 and Not1 enzyme. The σNS clone pCR vector clone digestion showed the σNS 1143 bp bands. pIRES vector digestion did not show enzymatic digestion bands on the gel.

4.1.3. Enzyme digestion analysis pIRES vector

The enzyme digestion analysis was conducted to determined that the pIRES vector have the correct size as described in the material and method section 3.6. After enzyme digestion the plasmid was run on 1 % agarose gel to determine the band size as shown in the Fig.A3. The plasmid was digested with Pst1, Hind3 and BamH1 vector cutting enzymes. The enzyme digestion of pIRES vector was successful and expected band size is shown on a 1% agarose gel.

Table.1. The digestion analysis of pIRES vector shows the plasmid, enzyme, buffer and expected band size.

Lane	Plasmid	Enzyme	Buffer	Expected band size
1	Ladder	-	-	-
2	pIRES	BamH1	NEB3	2283bp, 3809bp
3	pIRES	Pst1	NEB3	2426bp, 3666bp
4	pIRES	Hind3	NEB2	609bp,352bp,1328bp, 3819bp
5	pIRES	-ve Control	-	
6	Empty well	-	-	-

Lane1 has 1KB ladder and the lane 2 and 4 show significant digestion of plasmid and we get the expected band size as shown in the Table.1. In lane 3 digestion of plasmid was performed with Pst1 enzyme in the presence of NEB3, the vector was not completely digested and uncut vector was leftover as shown in Fig.A3. The lane 5 has negative control for uncut pIRES vector that show two bands, one represent the super-coiled and the other is relaxed plasmid.

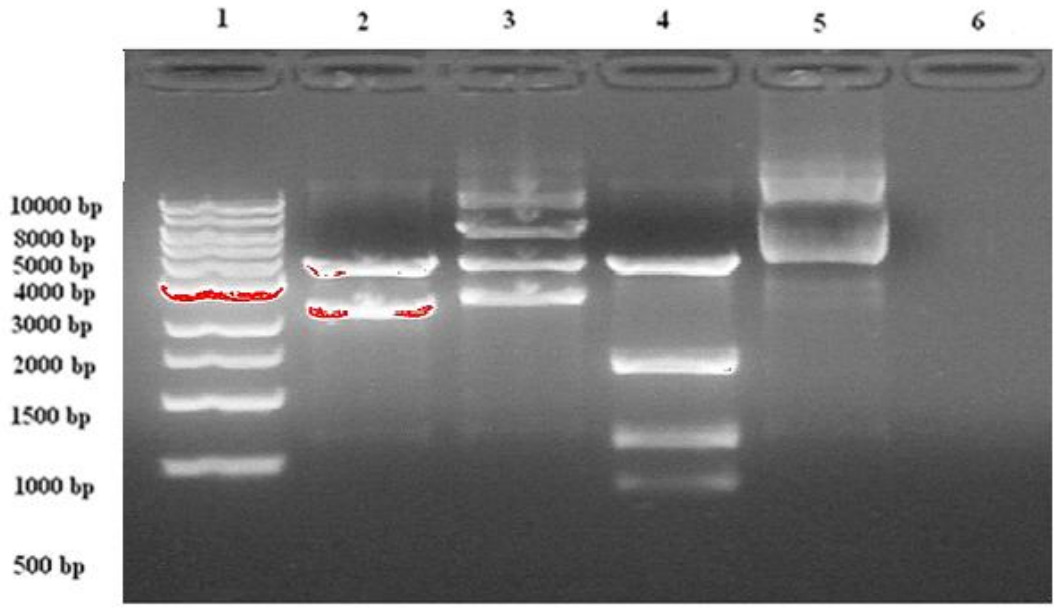

Figure.A3. Agarose gel (1 %) analysis of vector digestion (pIRES = 6.1KB). Lane: 1: 1KB ladder, 2: pIRES plasmid, NEB3 buffer and BamH1 enzyme, 3: pIRES plasmid, NEB3 buffer and Pst1 enzyme, 4: pIRES plasmid, NEB2 buffer and Hind3 enzyme, 5: negative control and 6: empty.

4.1.4. Isolation of linearized plasmid (pIRES) on low melting gel (0.75%)

The plasmid was treated with the Sal1 and Not1 to cut the vector at specific multiple cloning sites and to make the vector linearized as described in material and method section 3.6. It was an essential step before performing a cloning reaction. The linearized and non-linearized vector has not shown the expected band size as shown in the Fig.A4. Low melting gel (0.75%) was used to isolate pIRES vector and σNS gene PCR product. The blue arrow was drawn below the linearized vector in lane 4 and green arrow was drawn below the non-linearized pIRES vector in lane 6 as shown in the Fig.A4.

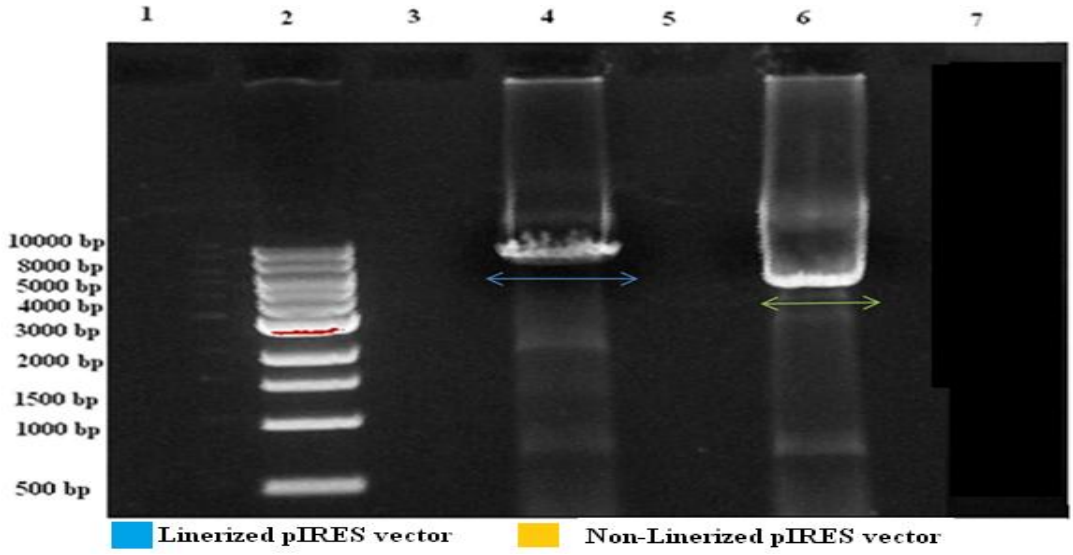

Figure.A4. Low melting gel (0.75%) used to isolate linearization pIRES vector (pIRES =6.1kb). The pIRES vector was cut and linearized Lane: 1 empty, 2: 1kb ladder, 3: empty, 4: pIRES plasmid, NEB3 buffer, Not1 and Pst1 enzyme, 5: empty, 6: pIRES plasmid, NEB3 buffer, 7: empty.

4.2. σNS-pcDNA 3.1 clone transfection

A σNS-pcDNA 3.1 clone was used to investigate the localization of the σNS protein in transfected CHSE cells according to Table.1.2. Before trasfection, the viability of cells was determined to 95 % and each millilitre contained 1.3 million cells as described in the material and method section 3.9.2.

Table.1.2. Transfection of σNS clone (pcDNA 3.1 vector containing σNS gene)

Well numbers 1-4	Well numbers 5-8	Well numbers 9-12
σNS clone	σNS clone	σNS clone
Sigma 1	Sigma 1	Sigma 1
Sigma 3	Sigma 3	Sigma 3
Positive control GFP	Positive control GFP	Positive control GFP

The isolated plasmid σNS-pcDNA 3.1 clone and clones of other PRV proteins (sigma 1 and sigma 3) in the same vector was transfected in the CHSE cells to determine the localization of

the σNS protein as described in the material and method section 3.9. It was found that the σNS protein localized to the cytoplasm and nucleus of the CHSE cells as shown in the figure.A6. The green fluorescent protein was expressed in CHSE cell as shown in the figure A6A. The primary mouse antibodies targeted the PRV gene σ3 as shown in the figure A6B and the σNS was also detected an antibody targeting the N-terminal Flag tag.

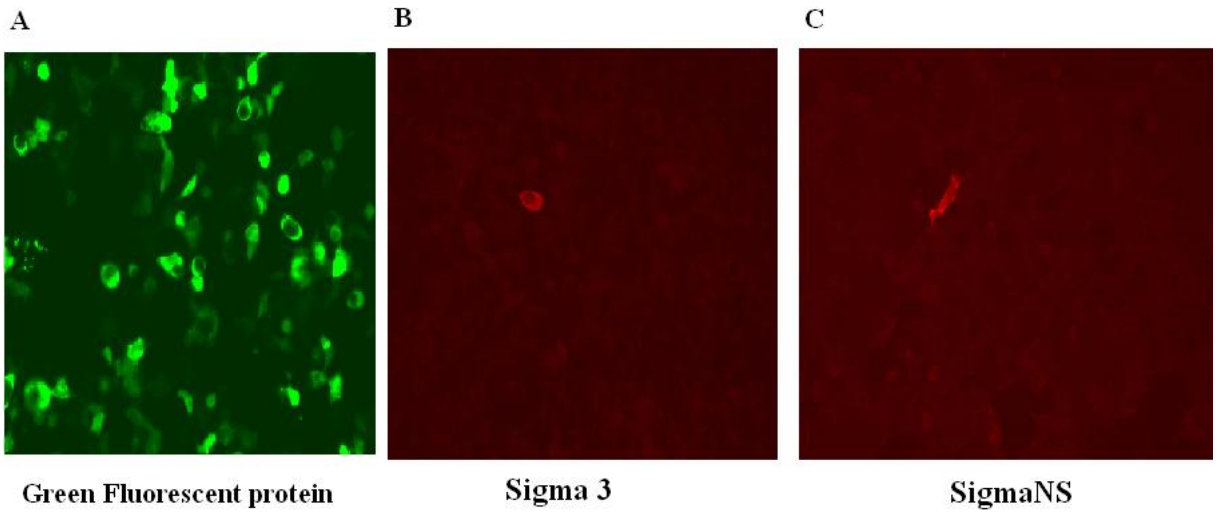

Figure.A6. Immunofluorescence and with fluorescence microscope. (A) The GFP protein was transfected as a control of expression in CHSE cells (B) The σ3 clone was transfected into CHSE cells and the σ3 protein was detected with specific rabbit antiserum (Finstad, NMBU) and secondary antibodies labelled with Alexa 488. (C) The σNS clone was transfected in CHSE cells and σNS protein was detected by a mouse anti-Flag antibody and a secondary antibody labelled with Alexa 595.

In a previous experiment performed by Maria K. Dahle the same σNS-pcDNA clone was transfected in CHSE cells and it was found that σ NS localized in the nucleus in just some of the transfected cells and the cytoplasm only in other CHSE cells as shown in the Fig.A7.

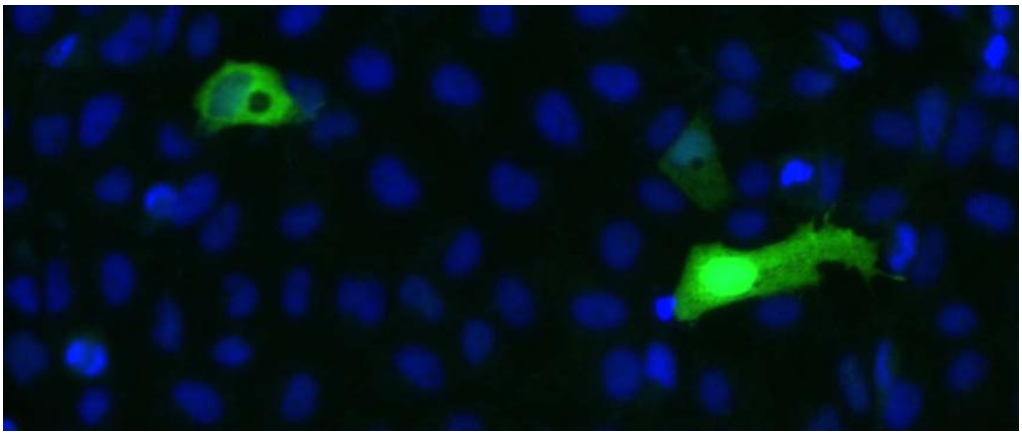

Figure.A7. The σNS-pcDNA clone that has Flag tag at the N-terminal was transfected in CHSE cells. The primary antibodies targeted the N-terminal Flag tag. The figure shows the localization of the σNS protein in both cytoplasm and nucleus of CHSE cells (Provided by Maria K. Dahle, unpublished results)

4.3. Expression of PRV and immune gene analysis in a fresh water cohabitant salmon study

4.3.1. Quantitative PRV-load in spleen tissues of virus injected salmon and cohabitant salmon

The PRV-σ3 gene copy numbers were quantified within cohabitant salmon (N=33) and virus-injected salmon (N= 12) by RT-qPCR as described in the material and method section 3.12. The arithmetical mean of the detected viral load (copy #) was presented in Fig.B1a. PRV was first detected in cohabitant salmon from WPC 6 and the samples from 0 to 4 WPC did not shown any PRV-load. It was found that spleen tissue sample of cohabitant salmon from WPC 8 had the highest PRV-load with approximately 100000 viral particles. The samples from WPC 6 have shown the approximately 13000 numbers of viral particles. It was estimated that cohabitant salmon from WPC 8 had 8 fold higher viral loads compared to the WPC 6. The PRV load was decreased 90 % in the cohabitant salmon at WPC 10 compared to the viral load of salmon from WPC 8. Moreover, PRV load had decreased 97 % in WPC 12 compared to WPC 8 samples. The statistical analysis indicates that standard deviation among WPC 8 samples was higher than rest of cohabitant salmon samples.

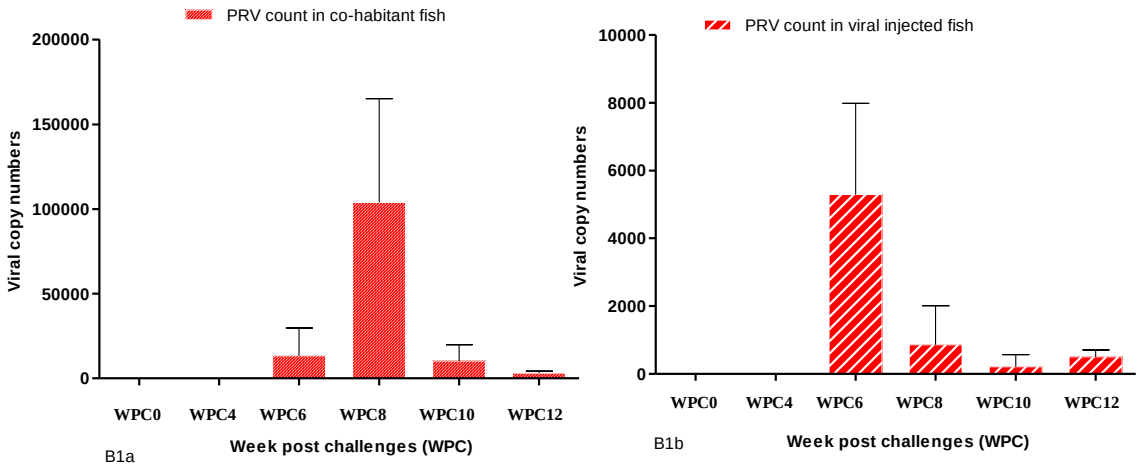

Figure. B1a &b. Detection of PRV load in cohabitant salmon (N=33 & N=12) by RT-qPCR in challenge study1 presented by mean (and SD) expression for spleen tissue samples. The spleen tissues of cohabitant salmon and virus-injected salmon of freshwater were used to quantify relative expression levels. The x-axis show the time point for sampling from week 0 to 12 post challenge and the y-axis bar show the number of viral targets (PRV).

The virus injected salmon of WPC 6 had the highest mean PRV-load with approximately 5000 viral particles that was decreased by 84 % and 96 % in WPC 8 and WPC 12 respectively as shown in the Fig. B1b. Moreover, PRV load was reduced by 94 % in samples from WPC 10 compared to samples from WPC 6 in viral injected salmon.

4.3.2. Quantitative and relative expression level of CD8α in spleen tissues of co-habitant and virus injected salmon of fresh water

The level of CD8α gene expression indicates the amount of cytotoxic T-cells (CTL) within the PRV infected Atlantic salmon. The relative expression level of the CD8α gene was measured in spleen tissues of co-habitant and virus injected groups of salmon. In this study elongation factor (EF-1a gene) expression level was used as reference gene for controlling up or down regulation of the immune genes analyzed (CD8α, granzyme & perforin). RNA was examined on a bio-analyzer to check the stability and to increase the validity of the present study as shown in the Fig.C3. The study samples were collected from six different points after virus injection (WPC 0, 4, 6, 8, 10, 12) and details about the samples were described in the material and method section 3.10. A tendency towards an increase CD8α relative expression

in samples from the cohabitant group WPC 4, 6 and 8 was observed as shown in the Fig. B2a. Also, a slight up regulation trend of CD8α relative expression was observed in virus injected salmon. The relative expression level of CD8α was approximately 2 fold higher in cohabitant salmon of WPC 8 and 10 respectively. However, the relative gene expression of CD8α was 2.4 fold higher in cohabitant salmon for WPC 12 as shown in the figure B2a.

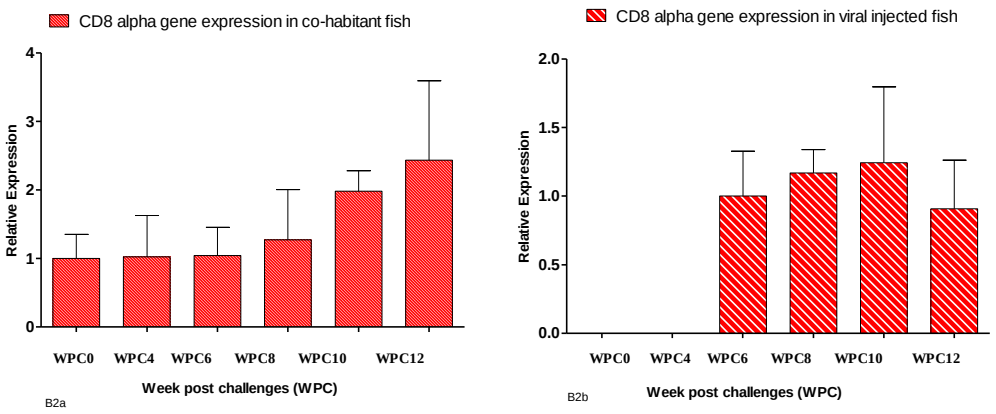

Figure. B2a & B2b. A quantitative gene expression of immune gene CD8α was measured with RT-qPCR and presented as relative expression changes at each time point. The spleen tissues of cohabitant fish (N=33 & N=12) of freshwater were used to quantify relative gene expression. The x-axis bar show the time point of sampling from week 0-12 post challenge and the y-axis bar show the fold change in relative expression level of the CD8α immune gene.

4.3.3. Quantitative and relative expression level of Granzyme in spleen tissues of co-habitant and virus injected salmon of fresh water

The granzymes are serine proteases which are released from cytoplasmic granules in Cytotoxic T cells and these granzymes activate the apoptosis (cell death) in the viral infected cells. The relative expression level of granzyme was quantified in spleen tissue samples of cohabitant and virus injected salmon collected at six different time points. The highest relative gene expression level of granzyme was found at WPC 8 and was 25 fold higher than the samples from WPC 0. The relative expression level was down regulated in WPC 10 and 12 compared to WPC8 in cohabitant salmon as shown in the figure B3a. The granzyme gene expression for WPC 10-12 was 13 and 8 fold higher respectively than WPC 0 in cohabitant salmon.

The spleen samples of WPC 6 have shown the highest granzyme expression among virus injected salmon samples. The granzyme expression level was sharply decreased by 95 % in the last week (WPC 12). Down regulation was also observed in WPC 8 and 10 of virus injected salmon samples.

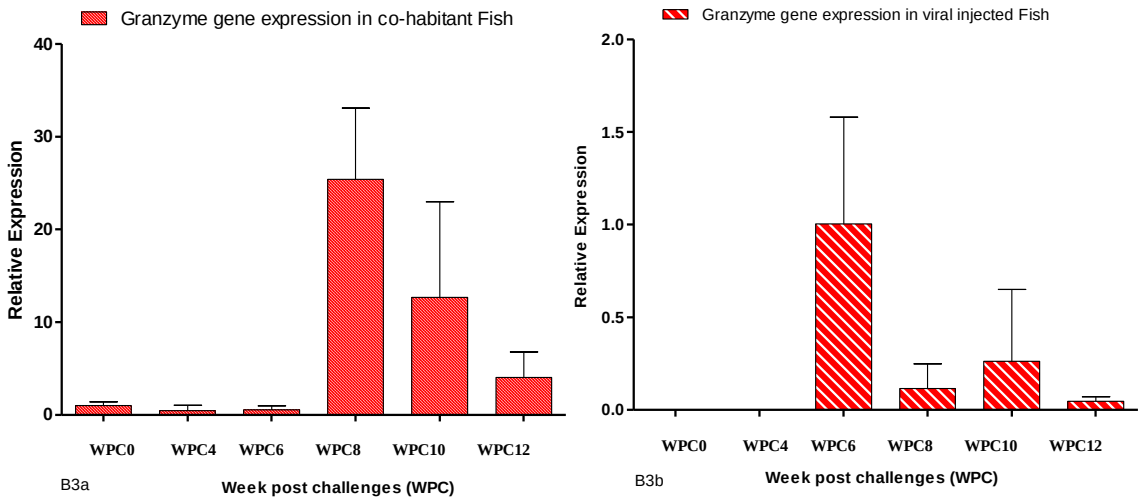

Figure. B3a & B3b. A quantitative gene expression of granzyme was measured with RT-qPCR and presented as relative expression compared to WPC 0 (a) or WPC 6 (b) (Set to 1). The spleen tissues of cohabitant or virus injected salmon (N=33 & N=12) of freshwater were used to quantify relative gene expression. X-axis bar show the time point samples from week 0 – 12 post challenge, and the y-axis bar show the fold change in relative expression level of the granzyme immune gene.

A standard deviation (S.D) among WPC 10 samples of cohabitant salmon was significantly higher (S.D ±9.41) compared to the cohabitant salmon from WPC 0.

4.3.4. Quantitative expression levels of perforin 1a and 1b genes in spleen tissue of Freshwater cohabitant salmon

Perforin is a protein present in the cytoplasmic granules of CD8 cytotoxic T lymphocytes. The perforin forms holes in the cell membrane of target cells. There are two variants of perforin 1 in salmon, 1a and 1b. The perforin 1a expression level was quantified in the spleen tissue of freshwater cohabitant salmon. The Ct-values were used to analyze the relative gene expression level at all-time point. At WPC 8 the highest perforin 1a expression level was shown which was 2 folds higher compare to the zero time point sample (Figure B4a). The

perforin 1a expression level was slightly lower at WPC 10 and WPC 12 compared to WPC8. The S.D. between samples from WPC 10 was higher than the rest of study samples.

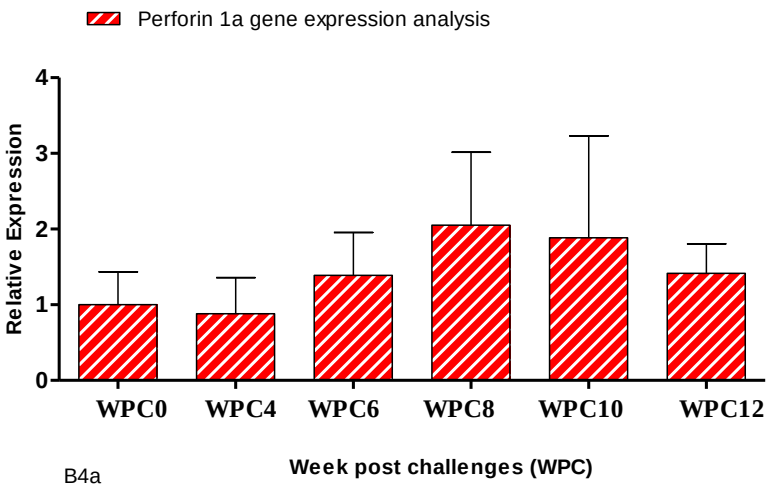

Figure.B4a. A quantitative expression level of perforin 1a was measured in spleen tissue of freshwater cohabitant salmon (N=33) with RT-qPCR and presented as relative expression at each time point. X-axis bar show the time point of sampling from week 0-12 post challenge, and the y-axis bar show the fold change in relative expression level of the perforin 1a.

The perforin 1b expression level was quantified in the spleen tissue of freshwater cohabitant salmon. The Ct-values were used to analyze the relative gene expression level at all-time points. The WPC 10 has shown the highest perforin 1b expression level that tended to be higher than zero time point samples (Figure.B4b). Perforin 1b expression only tended to increase compared to WPC 0 samples. The S.D. between samples from WPC 10 was highest ± 2.37 that was higher than the reset of study samples.

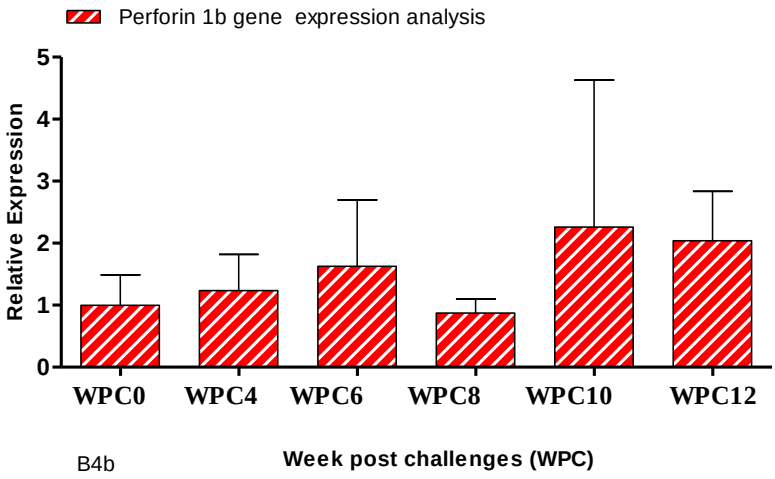

Figure.B4b. A quantitative expression level of perforin 1b was measured in spleen tissue of freshwater cohabitant salmon (N=33) with RT-qPCR and presented as relative expression at each time point. X-axis bar show the time point of sampling from week 0-12 post challenges, and the y-axis bar show the fold change in relative expression level of the perforin 1b.

4.4. PRV and immune gene analysis in a sea water salmon study

4.4.1. Quantitative and relative expression level of CD8 alpha in spleen tissues of co-habitant salmon of sea water

The CD8α gene expression level was quantified in the spleen tissue of saline water salmon and specifics about the samples are described in the material and method section 3.10. The elongation factor (EF-1a gene) expression level was used as reference gene for measuring up or down regulation of each immune gene (CD8α, granzyme & perforin). The Ct-values were used to analyze the relative gene expression level at all-time point (WPC 0, 4, 5, 6, 7, 8). At WPC 5 slight up regulation of the CD8α expression level was observed compared to zero time point sample. A tendency of slight down regulation of CD8α expression was observed in the WPC 7 and WPC 8 compared to the WPC 5 (Figure.C1). The standard deviation (S.D.) between samples from WPC 6 was highest ± 0.90 compare to the rest of spleen tissue samples of saline water salmon.

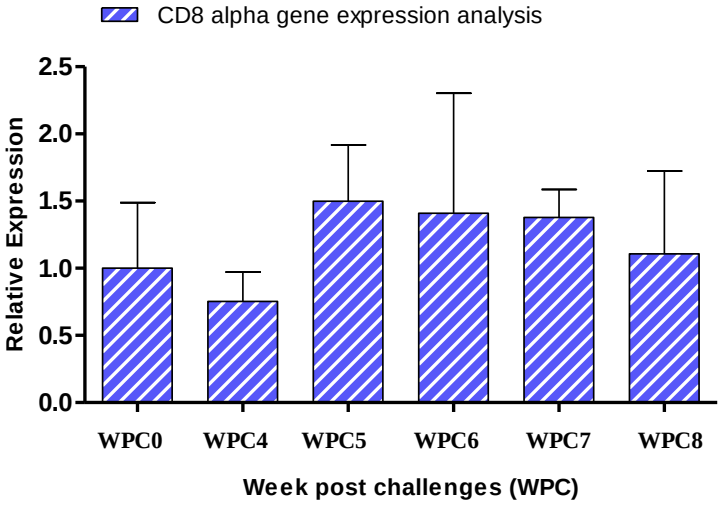

Figure.C1. A quantitative gene expression of immune gene CD8α was measured with RT-qPCR and presented as relative expression at each time point. The spleen tissues of cohabitant salmon (N=36) of salt water were used to quantify relative gene expression. X-axis bar show the time point sampling from week 0-8 post challenge and the y-axis bar show the fold change in relative expression level of the CD8α immune gene.

4.4.2. Quantitative and relative expression level of Granzyme in spleen tissues of co-habitant salmon of sea water

The granzyme gene expression level was quantified in the spleen tissue of saline water salmon. The Ct-values were used to analyze the relative gene expression in all time point samples (WPC 0, 4, 5, 6, 7, 8) and specifications about samples were described in the material and method section 3.10. The up regulation of granzyme expression stared WPC 5 which has shown 5 fold higher expression compare to WPC 0. The WPC 7 show the highest granzyme expression level that was 198 folds higher compared to zero time point samples as shown in the Fig.C2. WPC 6 showed slightly lower gene expression than WPC 7 but it was still 130 fold higher compare to WPC 0. The S.D. between samples from WPC 6 was highest ± 156.6 that was higher than the rest of spleen tissue samples of saline water salmon.

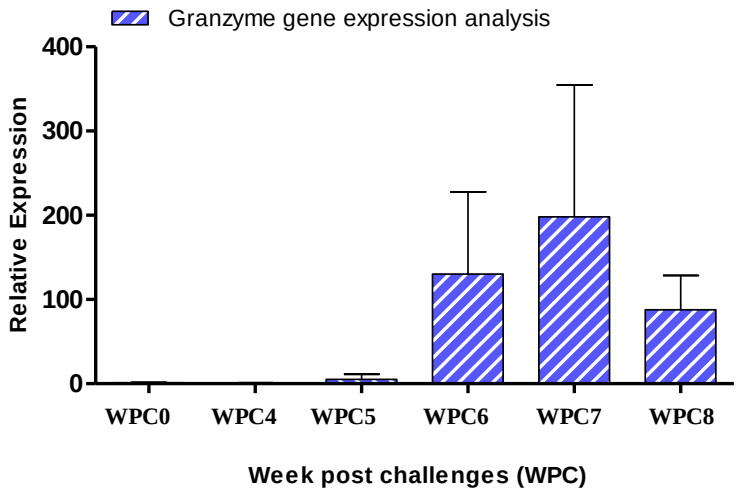

Figure.C2. A quantitative gene expression of Granzyme was measured with RT-qPCR and presented as relative expression at each time point. The spleen tissues of cohabitant salmon (N=36) of salt water were used to quantify relative gene expression. X-axis bar show the time point sampling from week 0-8 post challenge, and the y-axis bar show the fold change in relative expression level of the Granzyme immune gene.

4.4.3. Quantitative expression levels of perforin 1a and 1b gene in spleen tissue of sea water cohabitant salmon

The perforin 1a expression level was quantified in the spleen tissue of saline water salmon. The Ct-values were used to analyze the relative gene expression level between samples at all-time points. The WPC 6 & WPC7 showed the highest perforin 1a expression level that was 3 folds higher compare to the samples from zero time point as shown in the Fig.C3a. However, a trend of up regulation was also found in the WPC 8 compare to zero point samples. The S.D. between samples from WPC 6 was higheer ± 1.39 than the rest of study samples.

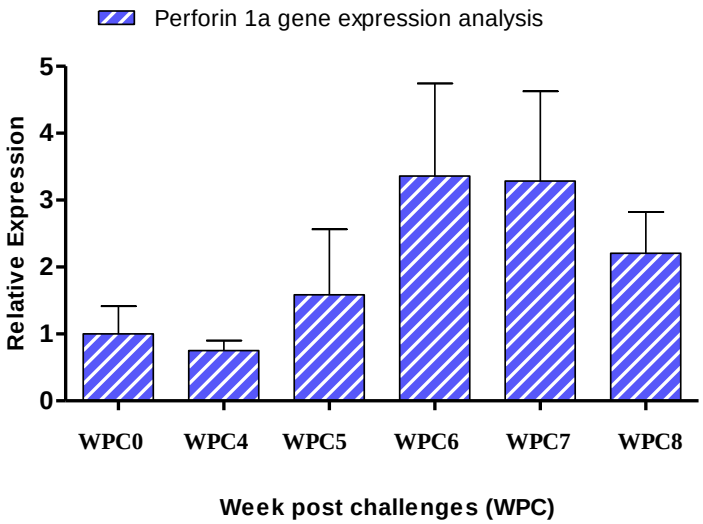

Figure.C3a. A quantitative gene expression of perforin 1a was measured with RT-qPCR and presented as relative expression at each time point. The spleen tissues of cohabitant salmon (N=36) of salt water were used to quantify relative gene expression. X-axis bar show the time point sampling from week 0-8 post challenge, and the y-axis bar show the fold change in relative expression level of the perforin 1a immune gene.

The perforin 1b expression level was also quantified in the spleen tissue of saline water salmon. The WPC 6 tended to have the highest perforin 1b expression level as shown in the Fig.C3b. The S.D. between samples from the WPC 7 was higher ± 0.85 than the rest of spleen samples of co-habitant salmon.

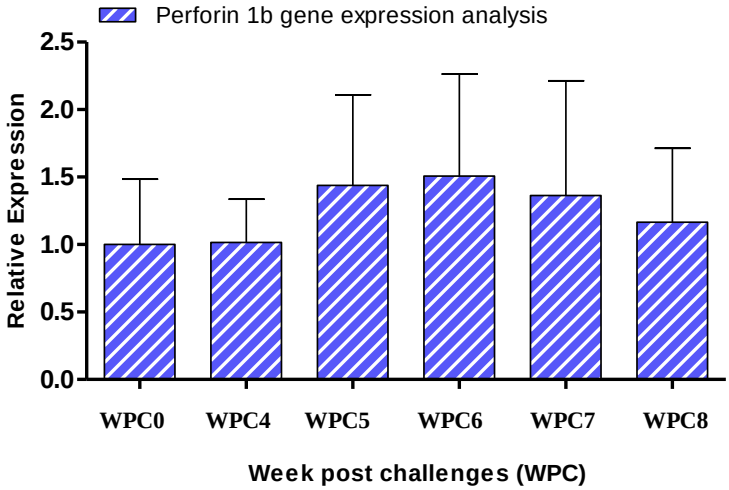

Figure.C3b. Gene expression of perforin 1b was measured with RT-qPCR and presented as relative expression at each time point. The spleen tissues of cohabitant salmon (N=36) of salt water were used to quantify relative gene expression. X-axis bar show the time point of sampling from week 0-8 post challenge, and the y-axis bar show the fold change in relative expression level of the perforin 1b immune gene.

5.0. Discussion

This master thesis project was conducted mainly with the aim to investigate the viral factory proteins for PRV and explore cytotoxic immune responses in infected Atlantic salmon. PRV have at least 10 different genes and two of them are the σNS and the μNS which are considered to play a significant role in the replication cycle and these two proteins are assumed to be involved in the formation of viral factories in the cell. It has been reported that the σNS and the μNS proteins from the related mammalian Orthoreovirus (MRV) are the minimal viral components required to form viral factories (Becker, M.M. et al., 2003) It was part of the project plan to clone these two genes (σNS and the μNS) in the bicistronic vector pIRES and transfect this clone into CHSE cell to determine the co-localization of these proteins. Immune fluorescence microscopy was to be used to determine the localization of σNS and μNS non-structural proteins. Confocal microscopy was also considered used to observe the formation of viral factories. In this thesis, only cloning and transfection of σNS was performed, and studied by immune fluorescence microscopy.

The analysis of challenge studies of freshwater and saltwater salmon were also included in this master thesis as an additional task. We quantified PRV load and measured the regulation of immune genes by using the RT-qPCR technique. The regulation of immune genes CD8α, granzyme, perforin 1a, perforin 1b and viral gene copy numbers were determined in viral injected and cohabitants salmons of freshwater. In addition, the regulation of immune gene CD8α, granzyme, perforin 1a and 1b were also measured in smoltified cohabitant salmons of a saltwater challenge study.

5.1 Methodological considerations

We attempted to amplify the PRV μNS gene several times with different primers using touchdown PCR and standard PCR techniques, but we failed to amplify the gene. The possible reason can be mutations in the gene sequence in the region where primers anneal to the template. Mutations at the primer binding region can potentially prevent the primers to anneal to the template and therefore fail to amplify a PCR product. Another possibility can be the denaturation temperature chosen for PCR amplification. A high denaturation temperature can be a solution for a gene that have a higher GC content in their gene sequence or form secondary structures. The μNS gene segments have an average GC contents in their sequence,

but their secondary structure is unknown. This possibility can be tested in order to amplify the μNS gene in a future study. However, degenerate primers can be a good option to amplify the μNS gene in case high denaturation temperature does not work.

Infusion cloning technique was selected to clone the μNS and the σNS genes into the pIRES vector. It was planned to clone the μNS gene with Myc tag in pIRES vector at multiple cloning sites (MCS)-A and σNS gene with Flag tag at MCS-B because the gene cloned into MCS-A would produce more protein, and more μNS protein than σNS protein is expected to be required to form viral factories. Infusion primers were designed to generate 15 extra base pair on both sides of the amplified μNS and σNS genes which were homologous with the linearized vector. This new technique is considered to be more accurate and easy than ligation-dependent cloning. The purified σNS sequence was ligated with linearized pIRES vector and was transformed in competent *E.coli* cells. The clones were tested by enzymatic digestion and by sequencing but we did not succeed to produce a positive clone.

Ligation dependent cloning was chosen in a second attempt to clone the σNS genes in pIRES vectors. We designed primers with additional Myc and Flag tag for the μNS and the σNS genes respectively and tried to amplify the PCR product of these two selected genes. The σNS-pCR clone was constructed using ligation dependent cloning technique and clones were sequenced to identify positive clone with complete σNS and myc tag sequence. One positive clone contained the gene sequence along with the myc tag at N-terminal. Some mutations were observed in the nucleotides of the σNS gene during sequence alignment compared with a previously sequenced σNS gene (Markussen, T., 2013) but it was found that these nucleotide mutations did not change the protein when we translated the nucleotide sequence into proteins in silico. Hence, they were silent mutations. Mutations could be due to use of different material for amplifying the gene (another HSMI outbreak, another year) and viruses commonly have small differences (polymorphisms).

Myc tag and Flag tag are polypeptides which were used to label the recombinant protein (σNS and μNS). These tags will helps in identification of protein among number of other proteins in the cell after transfection of the clone in the CHSE cells. Tag labelled protein can be isolated from the mixture of protein with the help of these tags. Moreover, antibodies synthesized against these tags can be used for immunological detection. These polypeptide tags are usually hydrophilic and exposed towards water. This localizes them to the protein surface and

makes them easily available to bind with the antibodies. On the other hand if we use hydrophobic tags it will be hard for antibodies to find their target to bind. The hydrophobic tags would probably hide inside the tertiary structure of the protein and be a difficult target for the antibodies to bind. On the other hand, hydrophilic tags can influence the protein function, for example by interfering with a protein binding site and the localization of the proteins in the cell. We have transfected the σNS-pcDNA clone into the CHSE cells two times and we found that in some cells σNS localized only to the cytoplasm, but in some cells it localized both in the cytoplasm and the nucleus. The reason behind the localization of σNS protein in the nucleus can be the amounts of over expression of protein in the cell which can disturb the normal localization. The CMV promoter of the pcDNA vector used is very strong, and will produce high levels of mRNA and proteins. There may also be other factors in the cell that control nuclear localization.

The immune gene expression level of salt water and fresh water salmon was tested by qPCR technique. The study samples of co-habitant and viral injected fish were not compared directly with each other because the analysis were run on separate plates, and the standard curve for each study group turned out quite different from each other. The reference study sample for co-habitant fish was set to WPC0, whereas the reference sample for viral injected fish was set to WPC6. However, relative immune gene expression data was compared within the group.

It is believed that qPCR is modern and sophisticated technique which provides valid information about gene expression but still there are some factors which can influence on the quality of data. These influential factors can be variation in individual handling, selection of experimental conditions and material, RNA quality and error at analytical phase.

5.2. Discussion of Results

Piscine orthoreovirus is considered to be the causative agent of HSMI in Atlantic salmon. It has 10 gene segments and two of them encode the non-structural proteins σNS and μNS, which are suggested to be involved in the formation of viral factories in the cells. In the present study immunofluorescence microscopy results indicate the localization of σNS in the cytoplasm and nucleus of CHSE cells. The localization of the σNS gene in cytoplasm and nucleus has shown that this protein does not form viral factories itself but it can be suggested

that σNS co-localization with the μNS in cytoplasm may form viral factories as it has been studied in other members of this viral family (Becker, M.M. et al., 2003). A σNS-pcDNA transfection in CHSE cells showed that σNS localized in the nucleus and cytoplasm of the CHSE cells, and that the localization differs between cells. It has been established that the function of σNS appears to be regulated by its subcellular and particular location and protein composition. Previous studies have shown the mammalian reovirus gene σNS distributed diffusely throughout the cytoplasm and did not form inclusion like structures (Becker, M.M. et al., 2003). The presence of functional σNS is required to form viral factories and it is not capable of developing inclusions in the absence of other viral proteins. These two non-structural proteins σNS and μNS are the minimum viral component required to form viral factories for MRV (Becker, M.M. et al., 2001). However, previous studies revealed that μNS is a major component of replication complex connected with MRV RNAs (Morgan, E.M., et al., 1975 & 77) and serve as a scaffolding protein that organizes other PRV proteins such as σNS and core particles during viral replication and assembly of viral factories (Becker, M.M. et al., 2003 & Broering, T.J.et al., 2000, 2002 & 2004 & Miller, C.L. et al., 2007 & 2003).

Early studies of mammalian reovirus has described that the viral factories formed only when functional σNS is present (Becker, M.M. et al., 2001) and it localizes to viral factories only in the presence of μNS. These viral factories are the sites of genome replication and progeny core assembly (Miller, C.L. et al., 2010). The mammalian reovirus (MRV) σNS has also shown expression diffusely throughout the cytoplasm when expressed alone in the absence of viral infection (Becker, M.M. et al., 2003). Previous studies have shown that non-structural proteins σNS and μNS of reovirus family are believed to be involved in the virus replication. Phylogenetic analysis of reoviridae has indicated that nonstructural protein of grass carp reovirus NS80 have conserved regions which indicates the special function of these regions in the viral replication cycle. The expression of non-structural protein in infected cells has been detected with the help of NS-80 specific antibody by immunofluorescence (Fan, C. et al., 2010).

The σNS was successfully amplified from material from a new HSMI outbreak in salmon. The σNS gene was cloned in the PCR vector and the PCR-σNS clones were sequenced to determine the correct sequence clone. The PCR-σNS sequence were aligned with the previously cloned and sequence σNS-pcDNA clone and obtain the correct clone as shown in the figure E6. We identified some mutation in nucleotides but these mutations were silent

mutation which did not change the amino acid sequence. The reason for these silent mutations can be the different DNA material which was obtained from different outbreak of HSMI among Atlantic salmon. PRV was completely sequenced in 2001 (Markussen, T., 2013).

A research trend to focus on early innate immune response studies viral infections in fish has been increased, as the response is a key factor to control the outcome of an infection. In this study, we used co-habitants salmon to demonstrate the transfer of PRV in spleen tissue of Atlantic salmon with HSMI and simultaneously we also studied the virus injected salmon to analyze the variation of immune response between these two study groups. The main reason we use spleen is that the virus infects blood cells, and the spleen is a very blood filled organ. It is also an organ that will accumulate infected cells over time. In the first study analyzed here blood was not available (Finstad, Ø.W. et al., 2014). The pattern of PRV transmission and variation in immune response was determined by quantifying the immune gene expression level. The cytotoxic T cells are responsible for killing the cancer cells and cells which are damaged or infected by viruses or intracellular organisms. These cytotoxic cells have cytotoxic receptors on their surface which bind to the infected cells. Antigens inside the infected cells bind with the class 1 MHC molecule in the cell and MHC with antigen is brought to the surface of the cell. These infected/damaged cells are recognized by T cells (CD8+ Tcells) which bind MHC1 with the help of glycoprotein CD8. These antigen presenting cells activate the T cells which have both CD8 alpha and beta receptors. Hence, upon viral exposure, the immune system can combat the viral invader with the help of CD8, and one gene is CD8α. In the present study, we determined the up-regulation and down regulation of immune genes. Previously, it was studied that the thymus, spleen and kidney tissues are the sources of CD8α expression in Rainbow Trout (Hansen, J.D. et al., 2000). The spleen is the tissue after thymus that has shown a significant expression level of CD8α and CD8β.The high relative expression level of CD8α has also been determined in spleen tissues of the Atlantic salmon (Moore, L.J. et al., 2005). The group of salmon with injected virus in our study showed only a slight up-regulation in expression of CD8α (Fig.B2). When virus is detected by the immune system the activated CTLs increased in numbers to combat the foreign invader. The activated CTLs have CD8α gene on their surface. We have determined a slightly elevated gene expression of CD8α along with the increase of viral number in co-habitant fish of fresh water. The up regulation of the CD8α also indicates that the viral exposure with the immune system which activates the cytotoxic T cells number. These

activated cytotoxic T cells bind with the infected or damage cell and secreted the granzyme in the infected cells to damage the cell.

The granzyme are conserved set of serine proteases which are found in human, mice and vertebrates but it's difficult to compare the fish granzyme with human and mice because it has not been well studied in fish (Grossman et al., 2003). The granzyme of Atlantic salmon has been sequenced (Gene bank no.NM-001141037). In present study the granzyme relative expression level have been found to be associated with the PRV load in the spleen tissue of the salmon. It was suggested that the viral load was directly proportional with the granzyme up-regulation because both were showing similar pattern of response in salmons of all WPCs (Fig.A2 & Fig.A3). Increased granzyme expression level has also been observed in the spleen of SVCV infected Cyprinus Carpio L. (Huang, R. et al., 2010). The relative granzyme expression level was low regulated in a spleen sample of co-habitant salmons during the first six week per challenges of the study but granzyme expression level was intensively increased up to 25 folds in cohabitant salmons from WPC 8. The granzyme relative expression level was decreased consistently in a salmon's spleen sample of next the following weeks. However, the virus injected salmon have different patterns of relative granzyme expression. The homogenate injected salmons showed the highest relative expression of granzyme at WPC 6 that was gradually decreased in salmons in the next week. The up-regulation of granzyme was also an indication of CTL activation; and these CTLs can bind to the MHC presenting cells. When the cells are infected and secrete granules of granzyme to in the target cells. These granules activate the caspases, inside the cytoplasm of target cells. The caspases may then to activate the mechanism of apoptosis (programmed cell death).

For the immune genes perforin 1a a slight gene expression up regulation was observed in both saltwater and freshwater challenge studies. A trend of higher perforin gene expression in later week indicates the higher number of CTLs in the immune system. Previous studies have shown that the CTLs directly attach with the infected/ damage cell and secrete the perforin to create the holes in infected cell. The CTLs kill the infected cell by perforin mediated pathway (Toda, H. et al, 2011). In present study, a trend of perforin up-regulation has shown association with the PRV viral load. The perforin gene expression was increased with the increase of viral load and vice versa. It indicates that perforin participate in immune defense to combat the viral infected cell by perforin mediated cytotoxic cell mechanism to kill the infected cell.

The viral infections are recognized for the high level of viral load variation between individual infected hosts (Wargo, A.R. et al, 2011 & Brault et al., 2011). In the present study, the quantitative PRVσ3 gene copy numbers were measured separately to determine the pattern of viral growth in both cohabitants and tissue virus salmons. It can be expected that virus spread first in the viral injected salmon then it transfer in the water through feces or other body secretions before it infected the other co-habitants fish. It can be predicted from current study that the PRV transmission from viral injected fish to healthy fish took 4 weeks after virus injection. The PRV load was first detected by RT-qPCR in WPC 6 of cohabitant fish. There is possibility that the viral transfers in cohabitant salmon occurs before WPC4 and accumulate in some other tissues which we have not analyzed. It was found that in cohabitant salmon, viral identification and multiplication was just started with salmon from the WPC 6. However, cohabitant salmon from WPC 8 contained a massive number of gene segments; those were 8 fold higher than the WPC 6 salmon and 33 fold higher than the WPC12 salmon. However, the time course of PRV load was quite different in the virus injected salmons, which showed the highest PRV load in salmon from WPC 6 that load was progressively decreased in salmons of the coming week. There was a possibility that the highest PRV load in salmon can be earlier than WPC 6 because we did not tested earlier samples for viral injected fish. The estimation of viral copies in another fish (yellow perch) spleen tissue was also used to establish a connection between time sequence and viral load, as the virus replicates more rapidly and significantly correlated within the spleen tissue (Olson, W. et al. 2013). In this study, the granzyme up regulation in spleen tissue was directly correlated with the viral load; both reached the highest level at the same week.

Fish immune system respond to infection in a differently from mammalians. Fish is a cold blood vertebrate which normally lives in a water temperature of 2-15 °C. Because of a lower body temperature the fish immune system develop antibodies against infection slower, during 6-8 weeks. However, mammalians have quite high body temperature and during infection the normal body temperature increase. The immune system activates and develops antibodies within 4-7 days after infection. The fish immune system do not form long lasting responses compared to mammalians. It has been reported that vaccine exposure in fish immune system developed immunity for 6 to 9 months and after this period immune system does not respond the same virus. The reason behind this immune system behaviour is still unknown. Moreover, the fish immune system respond to antibodies very slow and the body temperature can be the one reason among several others. It has been determined that HSMI infection occurrence is

more frequently in farmed fish than wild salmon. Probably there are several factors along with the viral infection which can be stressful environmental conditions, limited area to grow and feed for salmon. It is still unknown that how PRV transmitted from one salmon to other but like other fishes it is believe that faeces and other secretions can be the reason for transmitting PRV from one fish to other. It has been observed that the wild fish have healthy large intestine as compare to farmed fish which can be an entrance way for infection. It is still unclear that how PRV enter in the salmon.

HSMI is a new viral disease and very little is known about PRV and its mechanism of action. This research work on PRV is very distinctive in its nature because this is the first systematic study of immune responses in spleen, correlating with virus levels. One previous study has shown that HSMI is an infectious disease that can be transmitted via water in the cohabitant salmon (Kongtorp, R. T. 2006). On behalf of our results, we can suggest that the HSMI was transferred to the cohabitant salmons and up-regulated the expression level of immune related gene CD8α and granzyme.

5.3. Strength and weakness of present study

This study consists of two ports: one is based on cloning and the other is an immune gene expression analysis. The initial aim to the study was to determine the co-localization of σNS and μNS proteins and to clone them into the bicistronic pIRES vector, but this aim was not achieved. Moreover, we could not determine if the σNS protein localised to the cytoplasm or nucleus because in different cells we made different observations. The qPCR data of immune gene level of the co-habitant and viral injected salmon group could not be compared directly due to different standard curve that comparison can be helpful to establish an interesting association between viral injected and cohabitant salmons. The numbers of study samples per group were not sufficient to establish significant differences for some of the immune genes, even if tendencies of regulation were observed. The statistic tests (MANOVA, ANOVA and T-test) were not performed to determine the significant association of immune genes within the group and its association with the viral load.

5.4. Future perspective of the present study

The investigation of the PRV non-structural proteins σNS and μNS is necessary to investigate the infection mechanism of PRV in salmon because these two proteins actively participate in formation of viral factories in related viruses. The investigation of other PRV genes will help us to understand the mechanism of HSMI in salmon. There are several other factors which should be investigated to determine their association between PRV infection and the HSMI, including the effect of environmental conditions such as stress due handling and transport, rise and fall of water temperature, poor water quality including low oxygen levels in water, other infections and contents of fish feed. A large study will be performed at the Veterinary Institute and partner institutions in 2014-2017 to reveal the association between PRV and environmental factors for the development of HSMI in salmon. Moreover, development of new research tools, the development of vaccine against PRV, vaccine trials and their efficacy may limit the problem of HSMI in the futures.

6.0. Conclusion

- The σNS gene of PRV was successfully amplified and cloned in the pCR vector.
- The sigma NS gene (in pcDNA 3.1) was transfected in CHSE cells and the σNS protein localized to both the cytoplasm and the nucleus of the CHSE cells.
- In cohabitant salmon from challenge study 1, the viral count in spleen reached a maximum at 8 WPC in cohabitants, and the increase in granzyme correlated with the viral count.
- In viral injected salmon from challenge study 1, the viral count in spleen was highest WPC6, and also correlated with granzyme levels.
- In salmon challenge study 2, the cytotoxic immune response was highest at WPC6 & WPC7 in cohabitants, based on granzyme expression data.
- The markers of cytotoxic immune responses (primarily granzyme) increased when the virus levels increased in the cohabitants, indicating cytotoxic attack on virus infected cells.

7.0. References

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8.0 Publication

Characterization of cytotoxic immune markers in the spleen of Atlantic salmon infected with Piscineorthoreovirus (PRV)

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Abstract

Piscine orthoreovirus (PRV) is demonstrated to induce the disease Heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmosalar L*) smolts infected by experimental injection of infectious material or through cohabitation with infected fish. Natural HSMI outbreaks in farmed fish commonly occur some months after transfer to sea water pens, and PRV is found widely distributed also in asymptomatic farmed and wild salmonids. Lately, PRV have been reported found in salmon parr in fresh water cohorts in Norway, but few disease outbreaks have been reported at this stage, and the effects of the viral infection on parr has not been systematically studied.

In the present report, viral loads were assayed in tissue samples from a controlled PRV cohabitation challenge study in parr. The virus was found to accumulate in spleen for a limited time period after infection, and the peak viral loads in spleen were correlated with induced expression of genes linked to antiviral cytotoxic immune responses, an induction followed by strongly decreased PRV levels. Two different variants of perforin were induced. The parr in this particular study did not develop any pathological signs, and the findings indicate that immune cells in the spleen may effectively combat PRV infection through induction of cytotoxic activity.

9.0. Appendix

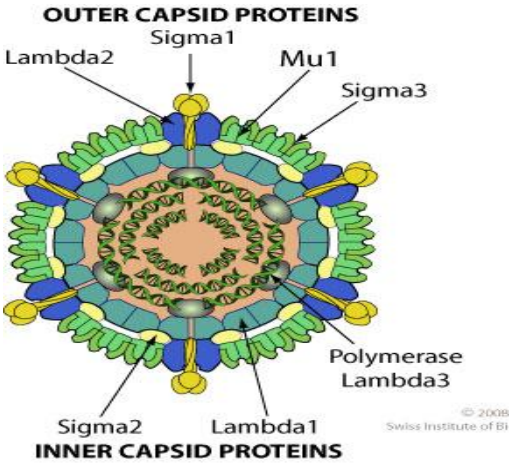


Figure.E1.A structure of Mammalian reovirus was developed that showed the inner capsid proteins and outer capsid proteins (Swiss institute of Biotechnology., 2008. http://viralzone.expasy.org/all_by_species/105.html)

σNS alignment

```
                1                                                                                                   100
MRV T3D σNS     MASSLRAAISKIKRDLVGQQVCPNYVMLRSSVTTKVV-RNVVEYQIRTGGFFSCLAMLRPLQYAKRERLLG-QRNLERISTRDILQTRDLHSLCMPTPDA
ARV-138 σNS     MDNTVRVGVSRNTSGAAGQTVFRNFYLLRCNISADGR-NATKAVQSHFPFLSRAVRCLSPLAAHCADRTLR-RDNVKQILTRELPFPSDLINYAHHVNSS
GCRV-873 NS38   MAHTGTATLINAERTDRTLRLLETFPSITITIRQDTSGRDLVSSVYSATGMSAAARNLNPIANVKNMRQPGYVKEAHTAINTVPLRVATSTGLELPTAHL
PRV σNS         -----MSNFDLGRQANKPKTEYHLNALPYLKCGIKNS-ESVGSVIINFPARFDTAKSVSPLSAMTNDGFLKFKDPSDSLASRDRPAFNDYVRALQFSPEH
                101                                                                                                 200
MRV T3D σNS     PMSNHQASTMRELICSYFKVDHADGLKYIPMDERYSPSSLARLFTMGMAGLHITTEPSVKR-VPIMHLAADIDCMILALPYMITLDGDTVVPVAPTLSAE
ARV-138 σNS     SLTTSQGVEAARLVAQVYGEQLSFDHVYPTGSATYCPGAIANAISRIMAGFVPHEGNDFTPDGAIDYLAADIVAYKFVLPYMLDIVDGRPQIVLPSHTVE
GCRV-873 NS38   ---AQMPVDQALRDAVAAAVPAHAARVLPPNVDRVTPLTLASRVAMVCAGLDCDDIHEIAP--APTAMA--LAFTIKVLLIHVVVDGTGASIAVNPGAAR
PRV σNS         -PHHFQALDPAFTDEILKTCDPTFNWTSIKSGDKYYLPAISQAIVYRASGFRFNSEKHLEQTGSLLPIALGISKATCALFVLVDSGT----VVCPEENVS
                201                                                                                                 300
MRV T3D σNS     QLLDDGLKGLACMDISYGCEVDANSRPAGDQSMDSSRCINELYCEETAEAICVLKTCLVLNCMQFKLEMDDLAHNAAELLKIQMMIPFSERVFRMASSFA
ARV-138 σNS     EMLTN-TSLLNSIDASFGIESRSDQRMTRDAAEMSSRSLNELEDHE-QRGRMPWKIMTAMFAAQLKVELDALADERVESCANAHVTSFGSRLFNQMSAEV
GCRV-873 NS38   DLLKA--DQLGNVITNMGYDVRGTVRRDTAAALAPSELPDTYPIEW-------LGLICGIIATQIELDLDMLAMNQTEQKLIAPHVQAVDPFINRLQSYA
PRV σNS         ALFSK--DKLSSLDIQFGYPKPKNGNDSTAYTKSINGYQIGAYGLK-LPGGHFLKLIHTLNCMCLKADLDLLSQVPSLADSLNRGMRCGYALLQYVSQFA
                301                                                                                 373
MRV T3D σNS     TIDAQCFRFCVMMKDKNLKIDMRETTRLWTRS-ASDDSVATSSLSISLDRGRWVAADASDARLLVFPIRV---
ARV-138 σNS     PIDRELMEIALIIKEQGFAMNPGQVASKWSLIRRSGPARPLAGARLEIRNGNWMIREGDQTLISVSPARMA--
GCRV-873 NS38   TLSSRLMHLCVTHAQRPFRD-FPELLRHWQKP----ELTPAIPVNIALKGAALEVSGNGAELFRVRALPIGGM
PRV σNS         TVDRELLLMSFLLKEANDPT-FHEVAAMWKSV--RDGTAQMDDVRFDLQPFGIMASTASLRDGVRIMAMFC--
```

Figure.E2. Multiple sequence alignment of PRV S3 ORF encoding the putative σNS protein aligned with σNS/NS38 proteins from reovirus prototype strains MRV T3D, ARV-138 and GCRV-873. Solid black lines represent sequence regions of higher conservation containing putative nuclear export signals. See text for more details (Markussen, T. et al., 2013).

μNS alignment

Figure.E3. Multiple sequence alignment of PRV M3 ORF encoding the putative μNS protein aligned with μNS/NS80 proteins from the reovirus prototype strains MRV T3D, ARV-138 and GCRV-873. ↓ = N-terminal end of second translation product of MRV (μNSC). Met-57, conserved in MRV and PRV is boxed red. ∗ = conserved putative zinc-hook motif crucial in the formation of inclusion-like structures in the MRV protein [86,90,152]. Black lines indicate sequence regions with higher level of conservation with the motif XGXDPX being boxed. In ARV, the larger region forms part of a region that has been shown to be involved in inclusion maturation [86]. Grey solid and dotted lines = coil-coil(s) regions as predicted by MultiCoil (window size: 21, probability cutoff: 0.5). The MRV L_{711}IDFS$_{715}$ motif shown to be required for the recruitment of clathrin to viral factories is boxed red (Markussen, T. et al., 2013).

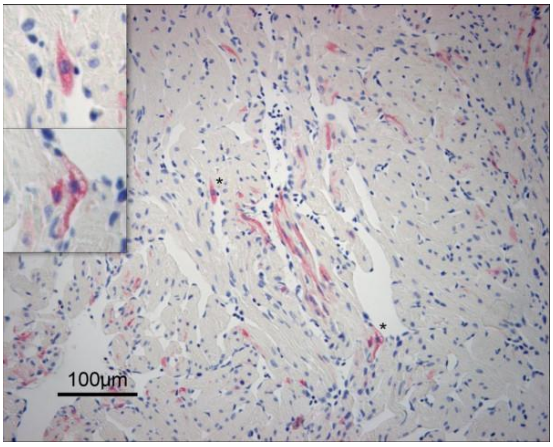


Fig.E4. PRV staining of cardiomyocytes. Immunostainingwith σ-1-antibodies of heart section from cohabitant group 10wpi. PRV antigen was detected in numerouscardiomyocytes in the ventricle (red color). This represents the time of peak staining in the cohabitant group and positive staining was observed in both the compact (right side) and spongy layer (mid to left side). Magnified sections (*) in the top left corner clearly show the cytoplasmiatic staining of the cardiomyocytes outlining the nucleus. Vocalization in positive stained cardiomyocytes is visible in the bottom magnified picture (Finstad, Ø,W. et al., 2012).

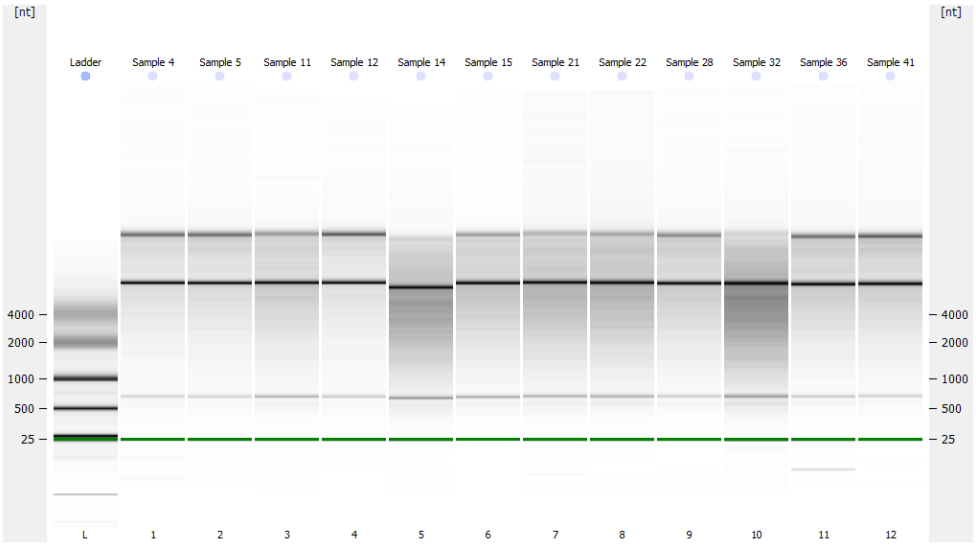
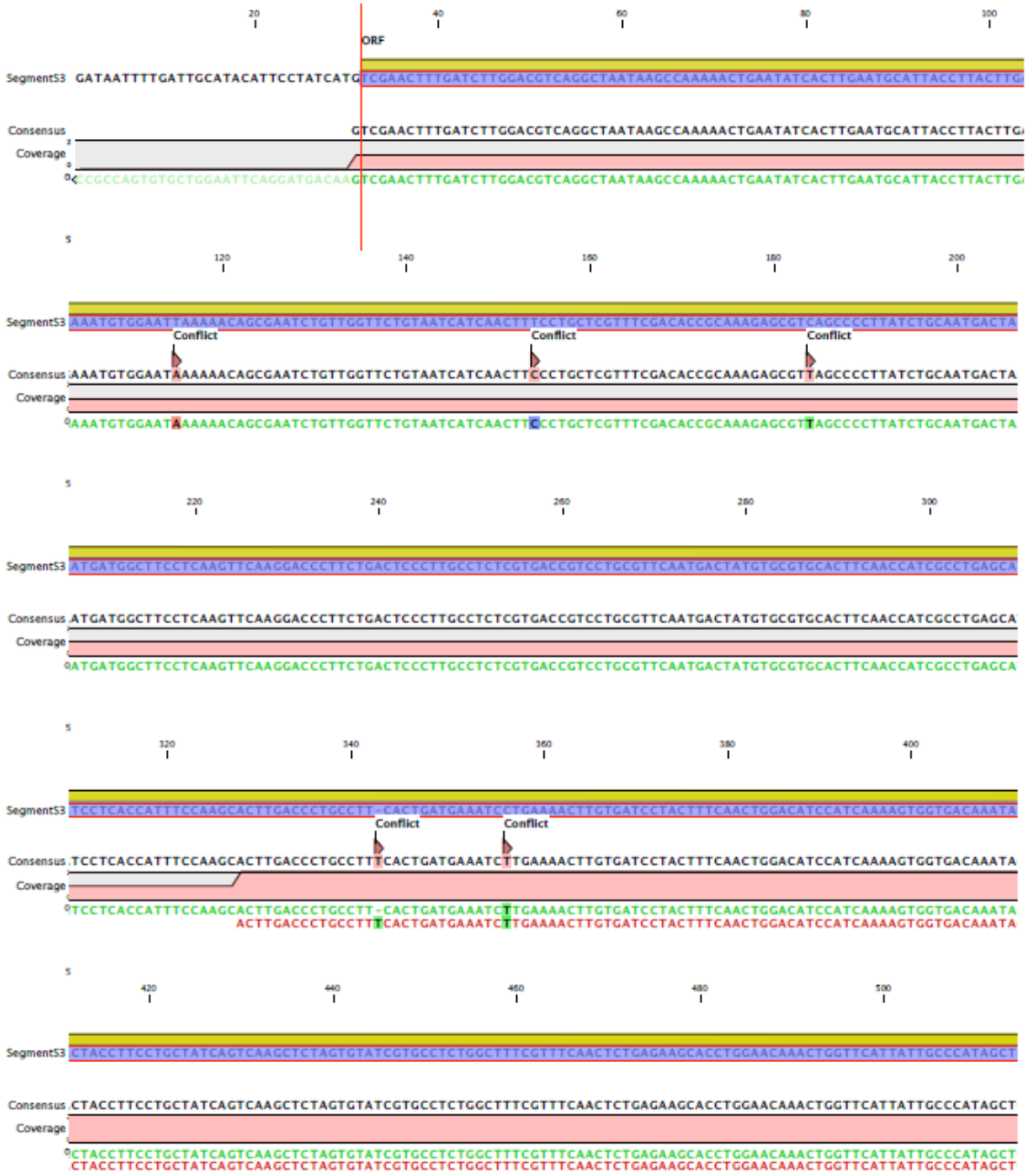
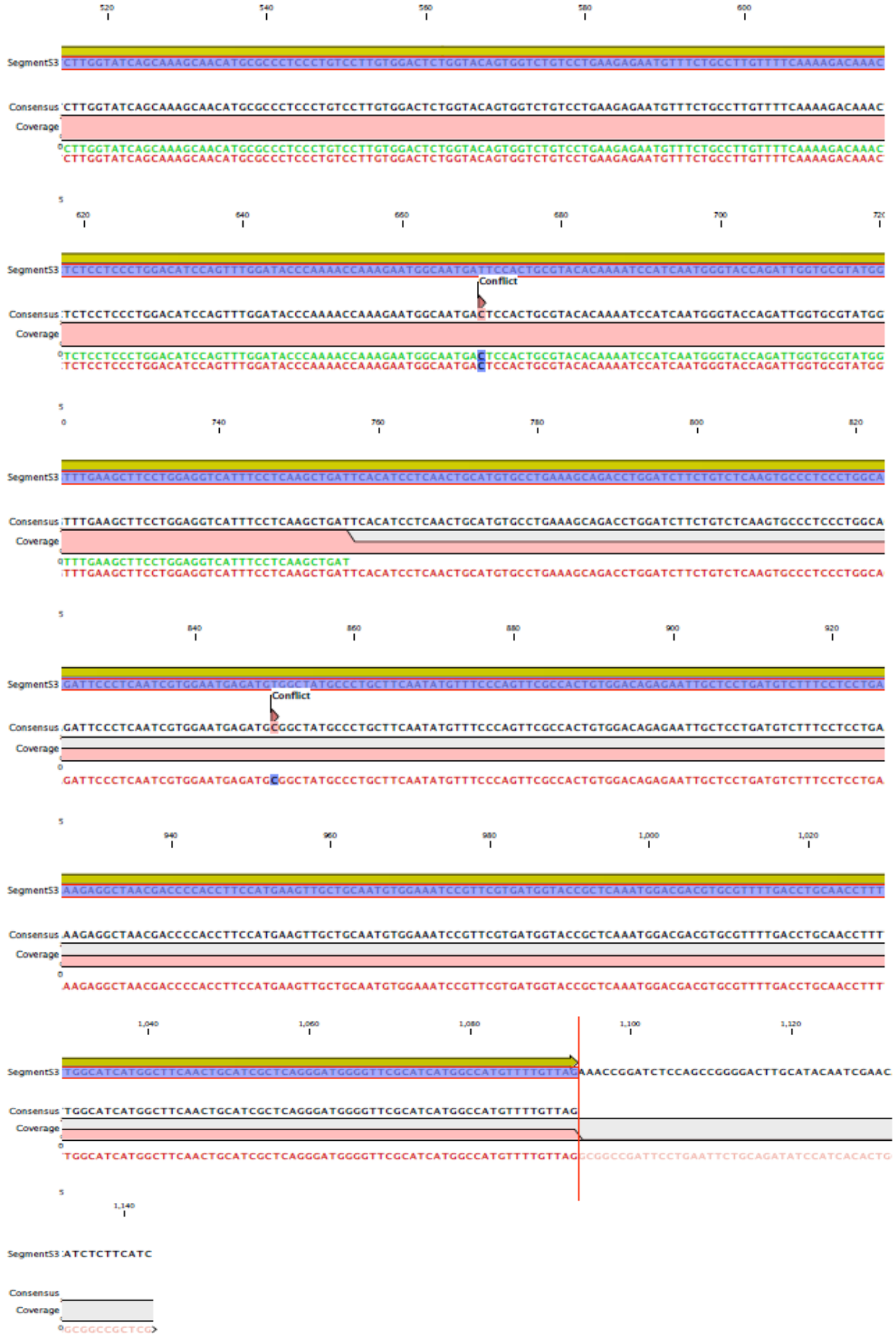


Figure.E5. RNA quality analysis. The assay was used to determine the quality of isolated RNA from spleen samples of cohabitant and virus injected salmons. A random selection of samples was made from each time span and a total of 12 samples were analyzed on Bio-analyzer.


ORF
Segment53
Consensus
Coverage
Conflict
Conflict
Conflict
Conflict
Conflict


520
540
560
580
600
SegmentS3
CTTGGTATCAGCAAAGCAACATGCGCCCTCCCTGTCCTTGTGGACTCTGGTACAGTGGTCTGTCCTGAAGAGAATGTTTCTGCCTTGTTTTCAAAAGACAAAC
Consensus
CTTGGTATCAGCAAAGCAACATGCGCCCTCCCTGTCCTTGTGGACTCTGGTACAGTGGTCTGTCCTGAAGAGAATGTTTCTGCCTTGTTTTCAAAAGACAAAC
Coverage
620
640
660
680
700
720
Conflict
TCTCCTCCCTGGACATCCAGTTTGGATACCCAAAACCAAAGAATGGCAATGACTCCACTGCGTACACAAAATCCATCAATGGGTACCAGATTGGTGCGTATGG
740
760
780
800
820
TTTGAAGCTTCCTGGAGGTCATTTCCTCAAGCTGATTCACATCCTCAACTGCATGTGCCTGAAAGCAGACCTGGATCTTCTGTCTCAAGTGCCCTCCCTGGCA
TTTGAAGCTTCCTGGAGGTCATTTCCTCAAGCTGAT
840
860
880
900
920
Conflict
GATTCCCTCAATCGTGGAATGAGATGCGGCTATGCCCTGCTTCAATATGTTTCCCAGTTCGCCACTGTGGACAGAGAATTGCTCCTGATGTCTTTCCTCCTGA
940
960
980
1,000
1,020
AAGAGGCTAACGACCCCACCTTCCATGAAGTTGCTGCAATGTGGAAATCCGTTCGTGATGGTACCGCTCAAATGGACGACGTGCGTTTTGACCTGCAACCTTT
1,040
1,060
1,080
1,100
1,120
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AAACCGGATCTCCAGCCGGGGACTTGCATACAATCGAAC
TGGCATCATGGCTTCAACTGCATCGCTCAGGGATGGGGTTCGCATCATGGCCATGTTTTGTTAG
GCGGCCGATTCCTGAATTCTGCAGATATCCATCACACTG
1,140
ATCTCTTCATC
GCGGCCGCTCG

Figure.E6. Genomic sequence Analysis of newly developed σNS clone with the old σNS clone to determine the mutation Genomic sequence were assembled to compare with the reference genomic sequence. Several conflicts were observed between newly cloned σNS gene and reference genomic sequence.

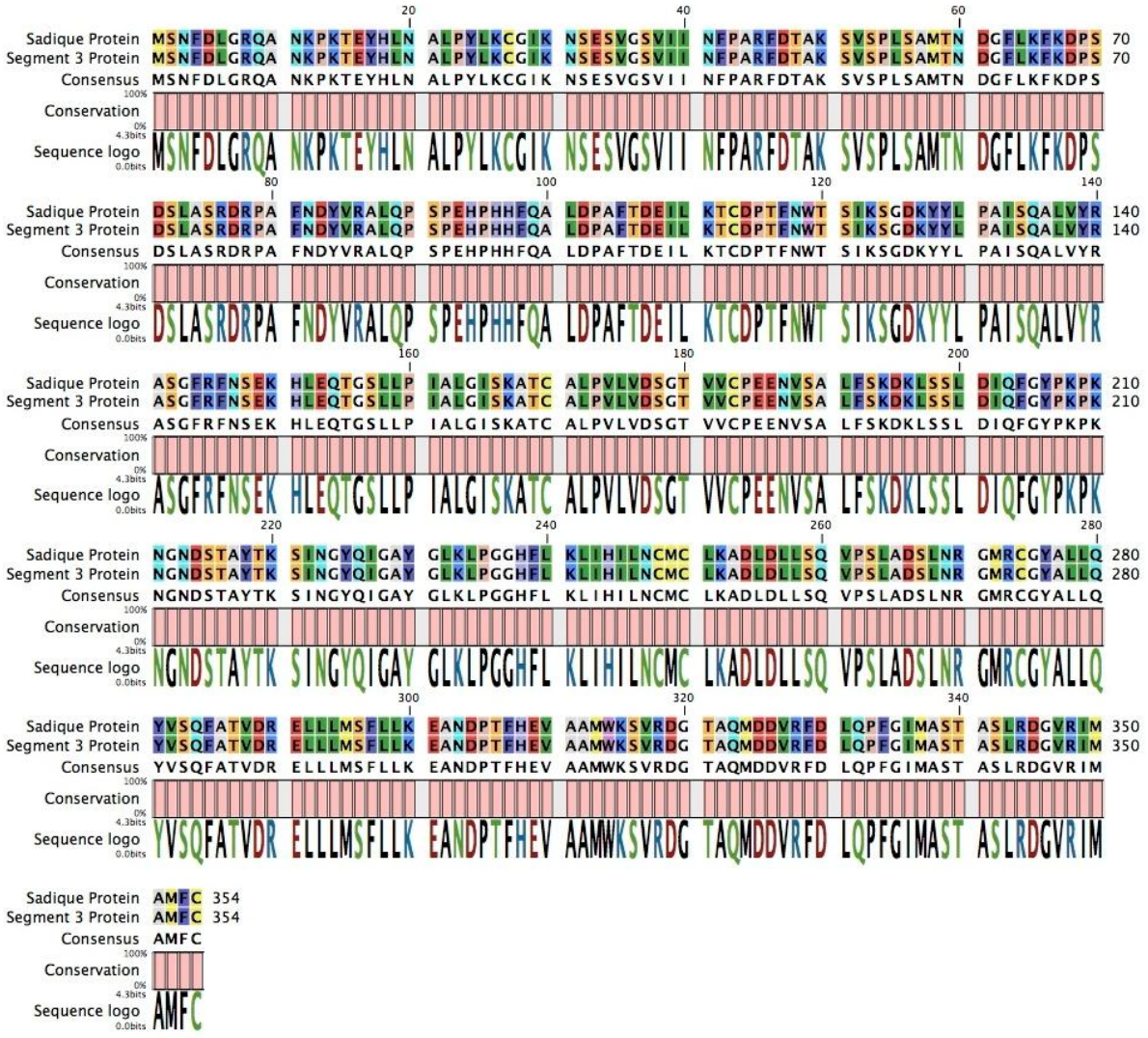

Figure.E7. Protein alignment Analysis to determine mutation: Protein sequences alignment was made between newly developed σNS clone with the old σNS clone to determine the mutation. Silent mutation was observed in newly cloned σNS gene during sequence alignment analysis.

Table.2. The name and sequence of the primers which were used to amplify the σNS and μNS gene.

Name of Primer	**Sequence**
pIRES-EcoRI-N-Flag-muNS-reverse	TCGACGCGTGAATTGCCACGTAGCACATTATTC
pIRES-EcoRI-N-Flag-muNS-forward	CTAGCCTCGAGAATTATGGATTACAAGGACGATGA
pIRES-BamHI-N-Myc-sigNS-forward	ATAGCGATAAGGATCATGGAACAAAAACTAATCTCAGAAGAAGACCTAATGTCGAACTTTGATCTTGGACGTC
pIRES-BamHI-N-Myc-sigNS-reverse	CACCATACGCGGATCCTAACAAAACATGGCCATGA

Table.3. The name of tag and sequence and molecular weight of sequence.

Tag name	**Sequence**	**Weight**
Flag tag	DYKDDDDK	1012 Da
Myc tag	EQKLISEEDL	1202 Da

Table.4. The primer sequences of immune genes, house hold gene and PRV σ3 gene (CD8α, EF1α, PRV-S1, Perforin 1a, perforin 1b and Granzyme) which were used to measure the gene expression.

Gene Name	Primer Sequence	Gene Bank no.
PRV-S1	Fwd TGC GTC CTG CGT ATG GCA CC Rev GGC TGG CAT GCC CGA ATA GCA Probe FAM- ATCACAACGCCTACCT–MGBNFQ	GU994022.1
EF1α	Fwd TGCCCCTCCAGGATGTCTAC Rev CACGGCCCACAGGTACTG	BG933897
CD8α	Fwd GTCTACAGCTGTGCATCAATCAA Rev GGCTGTGGTCATTGGTGTAGT	……
Perforin 1a	Fwd GCTACGTGAAGGTGGCTTTC Rev GGTTCCCAGGTCATAGCTCA	……
Perforin 1b	Fwd GCCAGTCGAAGAAGGACAAG Rev GAAGTGGTGCCAAAGTTGT	BT045592
Granzyme	Fwd TAAAGGTCGCATCCCTCATC Rev TCCAGACACTGAGCAGTTGG	NM-001141037

Table.5. Primers designed to amplify µNS with N-terminal Flag tag and σNS with C terminal Myc tag to clone into the pIRES vector at the EcoR1 and BamH1 sites.

Name of Primers	**Primer Sequences**
SP-σNS-Flag-Sal1	attgtcgacatgGATTACAAGGACGATGACAAGTCGAACTT TGATCTTGGACGTCAG
ASP-σNS -Not1	AatgcggccgcCTAACAAAACATGGCCATGATGCG
SP-µNS-N-Myc-Nhel	AttgctagcATGGAACAAAAACTAATCTCAGAAGAAGAC CTAGCTGAATCAATTACTTTGGAGGA
ASP-µNS-Xhol	tcgactcgagTCAGCCACGTAGCACATTATTCA

Table.6. The regents with given concentrations were used to produce cDNA from the spleen tissue RNA of infected Atlantic salmon.

Genomic DNA elimination reaction components

Component	**Volume/rx**	**Final [c]**
gDNA Wipeout Buffer (7X)	2 µL	1X
Template RNA (100 ng/µL)	10 µL	1 µg
RNase-free water	2 µL	---
Total volume	**14 µL**	

Reverse transcription reaction components

Component	**Volume/rx**	**Final [c]**
---*RT master mix*---		
Quantiscript Reverse Transcriptase	1 µL	---
Quantiscript RT Buffer (5X)	4 µL	1X
RT Primer mix	1 µL	---
---*Template RNA*---		
Entire gDNA elimination rx (step 4)	14 µL	1 µg
Total volume	**20 µL**	

Table.7. The name and sequences of primers which were used to sequence the σNS-pCR clone.

Name of Sequencing Primers	**Sequence**
T7 Fwd	TAATACGACTCACTATAGGG
M13 Rev	CAGGAAACAGCTATGAC