UiT The Arctic University of Norway

Faculty of bioscience, fisheries and economics

Specific antibody responses (IgM) against *Piscine orthoreovirus* (PRV) in Atlantic salmon (*Salmo salar*), measured in a multiplexed magnetic bead-based system

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Master's thesis in Aquamedicine (30 credits). May 2021.

Acknowledgment

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Lista, 15 May 2021

Monica Nordberg

Contents

Abstract:

Piscine orthoreovirus (PRV) causes heart- and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*). HSMI causes significant economic losses to the salmon aquaculture industry, and there is currently no vaccine available. In the trial this master thesis is based on, Atlantic salmon were injected with PRV-1, PRV-2, PRV-3 and inactivated PRV-1 to explore cross immunity and protection. In this thesis work, the focus was on detecting PRV specific antibodies (i.e., an IgM response). For the detection of PRV specific antibodies a multiplexed bead-based immunoassay was used to detect antibodies targeting the antigens PRV-1 σ1, PRV-μ1C, PRV-3 σ1 and PRV-3 μNS. PRV specific antibodies targeting PRV-1 σ1 were detected in Atlantic salmon immunized with PRV-2 and PRV-3, and PRV specific antibodies targeting PRV-1 σ1 and PRV-μ1C were detected in Atlantic salmon infected with PRV-1. There was also detected an increase in unspecific antibodies binding to controls after PRV-1 infection. No specific antibodies were detected in fish injected with InPRV-1. The PRV-3 μNS and PRV-3 σ1 antigens did not bind antibodies in this assay and could not be used as a reliable detection method. Immunization with PRV-3 induced anti-PRV antibodies and completely blocked a secondary PRV-1 infection and protected against HSMI. PRV-2 produced low levels of anti-PRV antibodies, but did not block PRV-1 infection or efficiently protected against HSMI. No antibodies were detected after immunization with inactivated PRV-1, but an intermediate protection was still obtained. Multiplexed bead-based immunoassay is a sensitive and fast method that can be used as a reliable diagnostic tool for immunity.

1.0 Introduction

1.1 Background

The Norwegian aquaculture industry has been the world-leading producer of Atlantic salmon (*Salmo salar L.*) for the last decade. The industry plays an important role for the global food market and the Norwegian economy, where approximately 95 percent of produced salmonids are exported (1). In 2020, Norway exported seafood for over 105 billion NOK (2), and in 2050 it is estimated that the Norwegian Aquaculture production will be traded at 550 billion NOK. However, there are many uncertain assumptions forming this prediction including fish health, disease and environmental challenges, innovation in breeding, cage engineering and fish feed (3).

According to the Norwegian Veterinary Institute (VI) the average mortality rate for farmed Atlantic salmon was 14.8 percent in 2020 and 16.1 percent in 2019 (4). A survey performed by "matfiskgenerasjoner" in 2011 also registered a total loss of 16.3 percent of farmed salmon from 288 locations in Norway. The latter survey concluded that the largest loss was due to infectious virus diseases (5). Another conducted survey from 2017, reported 53 million fish as lost in Norway, from which 88 percent (of the lost fish) died due to diseases and handling (6). One of the best measures against viral diseases in fish farming are vaccines. Most virus vaccines for fish in Norway are based on inactivated viruses and these have limited protection compared to vaccines directed against bacterial diseases. As a result, there is a need for more effective vaccines to be developed to combat pathogenic viruses in the salmon aquaculture industry (7).

The introduction of this master thesis is divided into three parts: A, B and C.

- Part A describes the situation of viral diseases in the Norwegian aquaculture industry, closing in on heart and skeletal muscle inflammation (HSMI) and vaccination.
- Part B describes the immune system of Atlantic salmon focusing on B-lymphocytes (B-cells) and antibodies.
- Part C describes the structure, subtypes, and immune responses to *Piscine orthoreovirus* (PRV).

Part A:

1.2 Viral diseases in the Norwegian aquaculture industry, focusing on Atlantic salmon

Viral diseases represent a large problem for the fish welfare and economy as it can increase mortality and reduce slaughter yield. If the Norwegian Aquaculture industry is to increase its production in a sustainably manner, solutions must be developed that prevent viral diseases from infecting Atlantic salmon (8). For the last two decades, the most common and serious viral diseases reported to affect Atlantic salmon have been heart and skeletal muscle inflammation (HSMI) with a disease outbreak of 161 per locality in 2020, pancreas disease (PD) with a disease outbreak of 158 per locality in 2020, cardiomyopathy syndrome (CMS) with a disease outbreak of 154 per locality in 2020, infectious salmon anemia (ISA) with a disease outbreak of 23 per locality in 2020, and infectious pancreatic necrosis (IPN) with a disease outbreak of 22 per locality in 2020 (9, 10).

HSMI caused by *Piscine orthoreovirus* subtype 1 (PRV-1) (11), PD caused by the *Salmonid alphavirus* (SAV) (12) and CMS caused by *Piscine myocarditis virus* (PMCV) (13) are all heart related diseases that commonly affect different regions of the heart of Atlantic salmon. HSMI is characterized by inflammation in the heart and red skeletal muscle, whereas the white muscle is unharmed to mildly infected (14, 15). For PD, the damage to the heart is mainly necrosis rather than inflammation, and in addition, most of its exocrine pancreas is missing (12, 16, 17). The pancreas is not damaged during HSMI, and by including the pancreas in the sample material for histopathology it is easy to distinguish between HSMI and PD (18). For CMS there are mostly observed degeneration and necrosis in the heart (19, 20). CMS can be distinguished from PD and HSMI since it normally causes changes in neither the pancreas nor skeletal muscle (19, 21). Histopathology of the heart might be challenging if co-infections of PD, CMS and HSMI occur in the same individual, where PD can mask histological HSMI and CMS, while HSMI can mask histological CMS. However, other methods like revers transcription real time polymerase chain reaction (RT-qPCR) are used to distinguish the viruses (22). ISA is caused by the *Infectious salmon anemia virus* (ISAV) and are related to circulatory disorder (ascites), and infects the endothelial cells causing severe anemia and pale organs (23, 24). Infectious pancreas necrosis (IPN) is caused by *Infectious pancreas necrosis virus* (IPNV) and infects exocrine pancreas and causes haemorrhagic enteritis (25, 26).

The Norwegian government has created measures to prevent the development of viral diseases in the aquaculture industry. These measures are described in regulations which includes prevention of outbreaks, minimizing outcome of outbreaks and fight against spread of contagious viral diseases (27, 28). One of the measures stated in the regulations is to report on any increased fish mortality or suspicion of disease to the Norwegian Food Safety Authority (FSA). As a result, the diseases can be detected early on and defeated at an early stage before they spread to adjacent farms or the environment (27). Another measure is the laws associated with the list of infectious diseases provided by the World Organization for Animal Health (OIE), diseases that are mandatory to report on to the FSA. There are three lists for infectious diseases. The European Union (EU) directive determines the diseases on list 1 (exotic diseases, e.g. epizootic haematopoietic necrosis) and list 2 (non-exotic diseases, e.g. ISA, viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN)), while the Norwegian government determines the diseases on list 3 (national diseases, e.g. PD) (29). Unlisted infectious diseases (HSMI, IPN,CMS) are not mandatory to report to FSA and are often left unreported when diagnosed by private companies.

The occurrence of viral diseases over the years is shown in fig.1.1 (9, 10). For the unlisted diseases, HMSI, CMS and IPN, there may be more outbreaks than reported in the yearly fish health report from VI. In 2020, the numbers of outbreaks reported by private laboratories was included in the outbreak collection examined at VI and resulted in an increase of reported outbreaks of HMSI and CMS. Therefore, the 2020-report, most likely, reflects the number of HSMI and CMS more truthfully than earlier years reports (10). Although the Norwegian government has strict regulations in regards to viral diseases within the aquaculture industry, companies often enforce additional actions to prevent disease outbreaks (30).

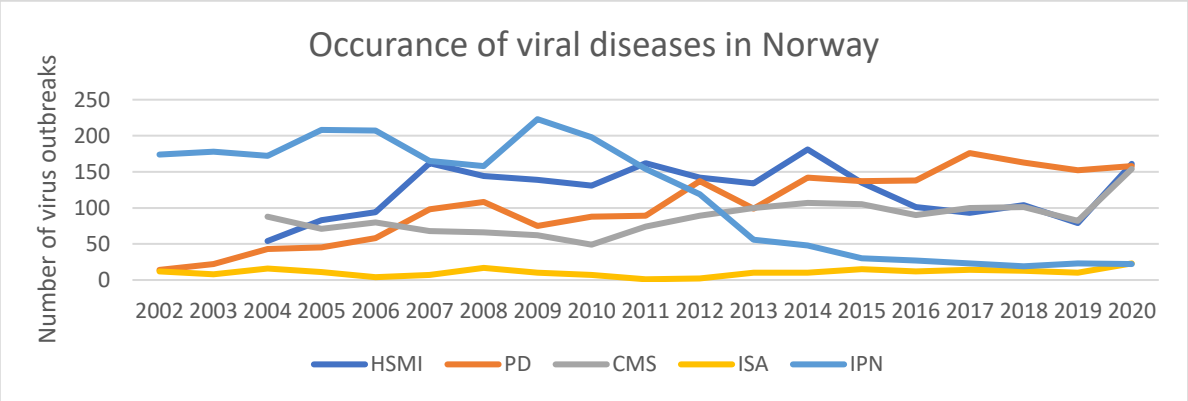

Figure 1.1. Development of HSMI, PD, CMS, ISA and IPN from 2002 – 2020. *From 2002 – 2019, not listed diseases are based on sample data examined by the veterinary institute (VI). For 2020, data from private laboratories are included with data from VI (9, 10).*

1.3 Heart and skeletal muscle inflammation (HSMI)

HSMI is a disease that affects Atlantic salmon, and was first diagnosed in 1999 (18), whereas the link between PRV-1 and HSMI was proven experimentally in 2017 (11). Mortality from HSMI usually varies from insignificant to 20 percent in farms (31), while the morbidity is almost 100 percent in affected cages (32). Stressors such as grading, delousing, and transport are reported to increase mortality of HSMI infected fish (31, 33). The disease is described to primarily harm the heart, in addition it cause inflammation to the skeletal muscle (11, 34). Atlantic salmon that die from HSMI often had significant circulatory disorders (ascites), pale heart with coagulated blood in the pericardial cavity, a large spleen and a grey film/veil (fibrinous layer) over the liver (fig.1.2) (18).

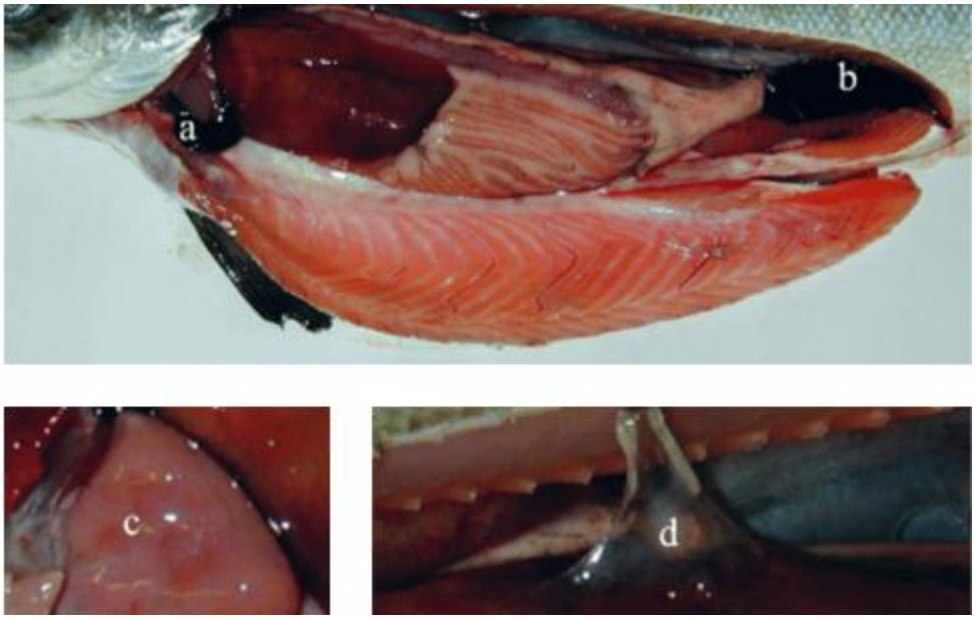

Figure 1.2. Heart and skeletal muscle inflammation (HSMI) in Atlantic salmon. *Macroscopic symptoms of HSMI in Atlantic salmon with a) pericardial cavity with coagulated blood, b) large/swollen spleen, c) pale heart, and d) fibrinous layer over liver. In addition to mentioned symptoms, blood or transparent liquid (ascites) are often observed in the abdominal cavity (14).*

PRV-1 is predominantly observed in farmed Atlantic salmon and to a much lower extent in wild Atlantic salmon (35). For farmed Atlantic salmon PRV-1 is mainly observed in the seawater phase (36), and is ubiquitous in the seawater phase in aquaculture, often without showing clinical signs (14, 36). However, PRV-1 has also emerged in freshwater facilities. Diagnostics of HSMI are therefore performed using histopathology as seen in fig. 1.3 (37). Common findings are inflammatory changes in the heart and in pronounced cases also in the red skeletal muscle (14, 38). For the heart pathology, the inflammation is more severe in the

epicardium and compact myocardium of the ventricle in early stages to a greater involvement of the spongy myocardium and atrium in later stages where it develops to panmyocarditis (14, 15). There are also observations of degeneration and necrosis with loss of transverse stripes. Atlantic salmon with pronounced cardiac pathology may show same type of degeneration of myocytes in the red skeletal muscle and degeneration and necrosis of hepatocytes (14). Persistent PRV-1 infection has also been reported to play a role in development of black spots (melanin) in the white skeletal muscle (39, 40), an economical problem as it downgrades the fillet quality (41).

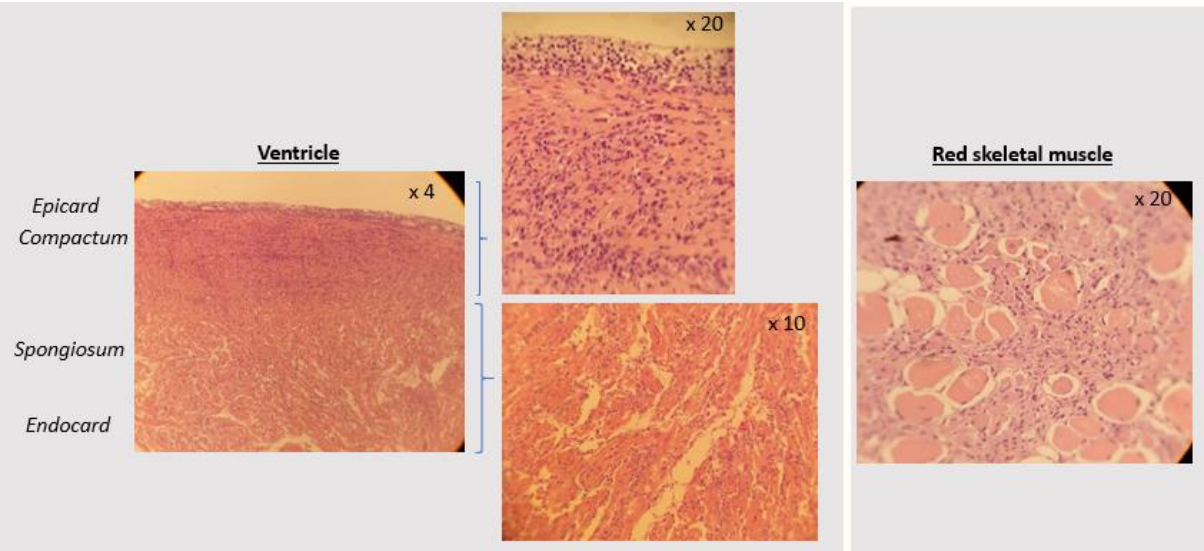

Figure 1.3. Histopathology of Atlantic salmon infected with heart and skeletal muscle inflammation (HSMI). *Histopathology of heart (ventricle) and red skeletal muscle of Atlantic salmon with HSMI showing massive inflammation in ventricle (specially epicard and compact myocardium) and red skeletal muscle. Histology pictures taken by Monica Nordberg during pathology course at UiT.*

Outbreaks of HSMI seems to be independent of region and type of farms, where some farms experience major problems with HSMI, while others experience few or no problems (33). There are indications of repeated HSMI outbreaks at some farms. This could be related to high stability of PRV-1 as it is a naked virus (no lipid membrane). This makes the virus more equipped to survive in the environment and more challenging to remove with wash (42). Another reason for repeated outbreaks may be related to the possibility of PRV-1 to circulate asymptomatically in the fish and then to infect other fish (33). The high prevalence of PRV-1 and most viral findings not being associated with clinical disease, led to HSMI being removed

from the OIE list in 2014 (33). There has been reports of functional feed and fatty acid component (tetradecylthioacetic acid) reducing the extent of HSMI, and hence increase survival during an outbreak (43, 44). However, this has not curbed the outbreaks of HSMI in Norway.

1.3.1 Current situation of HSMI

In 2020, data from private laboratories was included in the data from VI. Therefore, a higher number of HSMI outbreaks were reported than recent years (fig. 1.1) (9, 10). Data that are not reported represents a problem in relation to accuracy in occurrence and prevalence of the HSMI outbreaks.

HSMI outbreaks were reported on 161 locations with Atlantic salmon in 2020, where 153 outbreaks were reported from ongrowing farms, 7 outbreaks from smolt farms and 1 outbreak from a broodstock farm. In addition to this, there were PRV-1 detected on 89 farms without HSMI. From a survey completed by fish health specialists, HSMI was strongly associated with mortality at ongrowing farms, where only CMS was considered more serious when comparing virus diseases. HSMI was also related to reduced growth and welfare on ongrowing farms (45).

1.4 Vaccination of Atlantic salmon

Atlantic salmon is vaccinated before transfer to on-growing farms (46). Vaccination prepares the immune system against a particular disease by mimicking the pathogenic microorganism (47). There are different types of vaccines used in the aquaculture industry. In this section, attenuated and inactivated vaccination, delivery route and earlier PRV-vaccine trials will be covered.

1.4.1 Attenuated vaccines, inactivated vaccines and DNA vaccines

An attenuated live vaccine contains a weaker, non-pathogenic version of the pathogen. However, the pathogen is still able to enter and replicate in cells, and thereby trigger both the humoral and cellular part of the immune system. This results in a strong and long-lasting immune response in the host. Compared to inactivated vaccines, lower doses are required and there is no need for adjuvants and boosters. Nevertheless, there is a small risk of reversion to a more virulent pathogen due to replication (48).

An inactivated vaccine contains a non-living version of the pathogen that upon chemical or thermal treatment no longer replicate and cannot cause disease in its host (e.g., no risk of reversion). Inactivated vaccines are less efficient in providing an immune response and hence they cause lower protection in the host when compared to attenuated live vaccines (48). Some, but not all inactivated vaccines need booster and adjuvants to trigger the immune system to provide long lasting protection (49-51). Adjuvants improve immune responses towards vaccine antigens, where there are two types: depot (oil-based adjuvants) and immune stimulants (52).

DNA vaccines are genetic vaccines that encodes for one or several specific proteins (antigens) from a virus or bacteria to stimulate the immune system (53). A plasmid with encoded antigens is inserted into the muscle or skin and taken up by host cells which start producing the foreign proteins. The intracellular production of foreign proteins mimics an infection and the DNA can itself stimulate both the humoral and cellular part of the immune system offering long lasting immunity (54, 55).

1.4.2 Vaccine delivery routes

The three major ways of vaccine delivery are injection, oral and immersion. Oral vaccination is beneficial as it does not require handling of fish, but the amount of vaccine (feed) is difficult

to control. Amount of vaccine is also difficult to control during immersion vaccination as fish is kept dipped or bathed in the vaccine solution. An injection vaccine is administered to the fish either intramuscularly (i.m.) or intraperitoneally (i.p.) (fig. 1.4). There are major advantages using injectable fish vaccines such as longer protection period (over a year for some vaccines) (56), more than one antigen can be given in one shot (most complex vaccines given in aquaculture today contains up to 7 pathogens) (57), all fish are sure to get vaccinated and receive the correct dosage. However, injection requires handling and anaesthesia of the fish and thus increase stress (56).

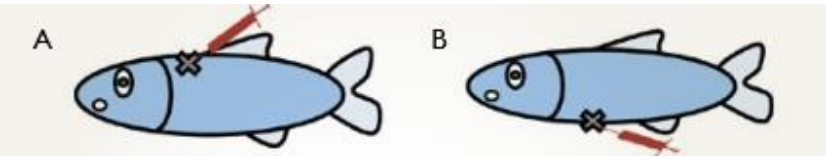

Figure 1.4. Injection of vaccine intermuscular (A) and intraperitoneal (B). *Retrieved from* https://www.hi.no/filarkiv/2016/03/laks_er_mer_utsatt_for_pd_nar_den_moter_virus_i_nye_farvann.pdf/nb-no

1.4.3 PRV vaccines

Presently, there are no commercial PRV-1 vaccines on the marked. The ongoing attempts to develop PRV-1 vaccines are demanding as PRV-1, cannot be cultivated and produced in available fish cell lines (58). In addition, PRV-1 establishes a persistent infection in Atlantic salmon, which increases the probability that PRV-1 may spread and mutate after vaccination (39, 59). Although there are challenges, two partly protective experimental vaccine candidates have been tested (60, 61). For the first vaccine candidate, PRV-1 was isolated from infected salmon erythrocytes, inactivated by formalin, and combined in an adjuvant formulation. The vaccine preparation and quality assurance were carried out by PHARMAQ AS. Upon i.p. injection in Atlantic salmon, this vaccine candidate demonstrated reduced heart lesions and viral loads in Atlantic salmon. However, it did not prevent PRV-1 infection (60). In the second vaccine trial, a DNA plasmid vaccine encoding the non-structural PRV-1 protein μNS, σNS and the cell attachment protein σ1 was i.m. injected into Atlantic salmon. The DNA vaccination delayed the infection of PRV-1 and induced moderate protection against HSMI. However, PRV-1 RNA levels in blood were not reduced, even though cardiac histopathology scores were reduced (61).

Part B:

1.5 Immune system – an overview

The main task of the immune system is to maintain homeostasis and protect the host against foreign materials and pathogenic microorganisms. To eliminate foreign invaders, it is crucial that the immune system can recognise self from non-self. The immune system is comprised by lymphoid organs forming defined anatomic sites that are dispersed through the body. At these sites, the immune cells develop from stem cells into mature immune cells, that are competent to respond to antigens. And then, upon encountering antigens the matured immune cells become functionally activated. The lymphoid organs are divided into the primary organs, which are the producers of T-lymphocytes (T-cells) and B-lymphocytes (B-cells), and the secondary organs, where immune responses are initiated through uptake of antigens and interaction between innate and adaptive immune cells (47). Teleost lacks bone marrow and lymph nodes that serves as lymphoid organs in mammals. Instead, thymus and head kidney (HK) function as their primary organs, while the kidney (including HK), spleen and mucosal associated lymphoid tissue (MALT) are their secondary lymphoid organs. The HK consist of hemopoietic tissue, developing B-cells and has antigen sampling ability. The thymus consist of developing T-cells (62).

1.5.1 The physical and chemical barrier – the body's first line of defence

The physical barriers of the fish are the skin, gills, and gut. They prevent infections by blocking the pathogens from entering. The barrier contains mucus with antimicrobial peptides/molecules and immunoglobulin (Ig) secreted by MALT that capture, alert the immune system and kill the microorganisms (63).

1.5.2 The innate immune system

The innate immune system provides the first, immediate response to an infection, but does not remember prior encounters with a pathogen. The innate immune system consists of macrophages/monocytes[1], dendritic cells (DC-cells), Natural Killer cells (NK-cells) and

[1] Develop from monocytes (patrols the blood) to macrophages (stationed in the tissue) 64. Lea T. Immunologi og immunologiske teknikker: Fagbokforlaget Vigmostad & Bjørke AS; 2006. 393 p..

granulocytes (neutrophil, eosinophil, basophile). The system is activated when cells of the innate immune system recognize pathogen associated molecule patterns (PAMPs) on the microorganisms. These patterns are essential for survival and infectivity of microorganisms and represents a large variety of molecular signatures that has been conserved though evolution (i.e. ss/ds RNA, CpG DNA, flagella). The PAMPs are recognized by pattern recognition receptors (PRRs). PRRs are evolutionary ancient receptors expressed by innate immune cells, of which some are expressed on B-cells. PRRs are non-clonal (identical receptors on all cells of the same lineage), and includes RIG-like receptors (RLR), Toll-like receptors (TLR) and Nod-like receptors (NLR) that are all identified in Atlantic salmon and represent a greater expansion of PRR families than in mammals (47, 65-67). Upon PAMPs recognition, most PRRs leads to upregulation of various immunogens, including cytokines, chemokines and type I interferons (IFN) (47).

Phagocytosis:

Phagocytes are cells performing phagocytosis. Phagocytosis is a special form of endocytosis where the pathogen is engulfed, fused together with lysosomes containing hydrolytic enzymes (i.e. lysozyme) and broken down (47). The phagocytic cells in salmonids are the DC-like cells (68-70), macrophages, neutrophils (71-73), and phagocytic B-cells (74-76). The capability of phagocytosis by B-cell varies between teleost species and are relativly high in Atlantic salmon compared to Atlantic cod (*Gardus morhua*) (76).

Degranulation:

Degranulation is a strategy where reactive substances are released from cells. One type of degranulation is release of perforins and granzymes by natural killer (NK) cells or cytotoxic T cells to trigger apoptosis in virus infected cells (47). Non-specific cytotoxic cells (NCC) are identified in teleost and are seen as the evolutionary precursor of NK cells (Greenlee et al., 1991). The NCCs spontaneous kills infectious pathogens that infiltrates the fish without any activation forehand (77).

Opsonization and complement activation:

Opsonization is a process where microorganisms are tagged for recognition. Antibodies, complement factors and soluble recognition molecules (i.e. pentraxins and collectins) opsonise microorganism. This facilitate the binding to specific receptors on the phagocytes. where they induce a cellular response (phagocytosis, degranulation, activation of complement system. There are three complement pathways: named the alternative, the classic and the lectin. The complement system consists of series of complement proteins, where cleavage of one protein result in a cascade of reaction. The activation of these pathways either opsonize or result in lysis of pathogens by forming membrane attack complexes (MACs). Alternative pathway is activated by complement protein 3 (C3) which are cleaved to C3b and have high affinity to pathogenic surfaces (47). To activate the classical pathway, antibodies (IgM) are required. IgM can be produced before or after antigen stimulation (78). IgM bind to pathogen and bind complement protein 1 (C1). The lectin pathway recognizes carbohydrates (mannose) on bacteria and bind mannose-binding-lectin (MBL), activating the complement system (47).

1.5.3 The adaptive immune system

Teleost lack lymph nodes, follicular structures, and germinal centre (GC) (79), and there is no class switching and a modest antibody affinity maturation. Teleost therefore rely on the innate immune system for an extended period until the adaptive immunity is kicked off (80, 81). The adaptive immune system consists of T-cells and B-cells. These lymphocytes recognize antigens ty heir T-cell receptor (TCR) and B-cell receptor (BCR). Each TCR and BCR are unique since they are obtained by point mutations (AID) and somatic recombination of gene segments (VDJ genes) where recombination activating genes 1/2 (RAG 1/2) are central. When the correct antigen is recognized by BCR and TCR, the cells after additional cytokine signals can develop from naïve to activated B-cells and T-cells (47).

1.6 Cell-mediated adaptive immune responses

Cell-mediated adaptive immunity is organized by T-cells with their TCR complex. The TCR interacts with cells though Major Histocompatibility Complex (MHC) (47). Teleosts possess αβ TCR and γδ TCR, where the latter is not well studied in teleosts (82). Most T-cells are αβ TCR and divided into two groups: T-helper (Th) lymphocytes (CD4+) and T-cytotoxic (CD8+) lymphocytes (CTL). CTL recognize endogenous antigens and Th-cells recognize exogenous antigens, by interacting with MHCI and MHCII, respectively (83-85). MHCI are expressed by all cells, while MHCII are expressed by so-called antigen presenting cells (APC). APCs in salmonids include macrophages, B-cells, and DC-like cells. The latter share characteristics with the mammalian DCs and are so called professional APC and have the ability to present antigen and activate naïve T-cells (70, 86-88).

Th-cells (CD4+) can be activated by APC where they, in mammals, differentiate into Th1, Th2 or Th17. Th1, Th2 and Th17 secrete cytokines that target host defence against intracellular pathogens, parasites and extracellular pathogens, respectively (47). Teleosts secrete cytokines that are signatures for Th1, Th2 and Th17 in mammals. This indicates that different Th populations resemble those in mammals (89). Teleost possesses cytotoxic cells that are functionally equivalent to CTL (CD8+) in mammals (83, 89). CTL (CD8+) are effector cells that causes cytotoxic responses. The TCR and CD8 interacts with MHCI and kills virus infected cells and cancer cells either through FAS ligand or degranulation (47).

In non-vaccinated salmonids, mature T-cells are abundant in thymus, MALT (gills and intestine) and in the interbranchial lymphoid tissue in the gills. In addition, lower number of mature T-cells are dispersed throughout the body of salmonids in organs such as kidney, spleen, peripheral blood (PBL), liver and heart (90, 91). Activated T-cells can differentiate into “long lived” memory cells that survive post-infection. Memory T-cells are reported in kidney of rainbow trout (92), but more research is still needed.

1.7 Humoral adaptive immune responses

Humoral adaptive immune responses consist of antibodies secreted by plasma cells. B-cells differentiate to plasma cells upon binding of antigens with their B-cell receptor (BCR), a membrane-bound immunoglobulin (Ig). Antibodies and BCRs consist of an Fc-part and a Fab-part (fig.1.5). The variable region of the Fab-part binds to the antigen, while the Fc-part binds to phagocytic and cytolytic cells and to different immune effector molecules (47).

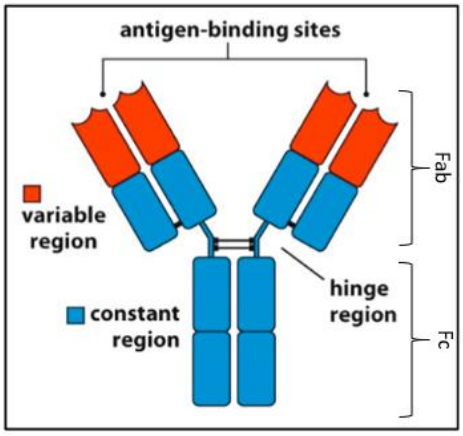

Figure 1.5. The structure of an antibody and BCR (Ig). *The Fab-part with one variable and one constant region. The Fc-part with two constant regions. Retrieved from https://www.memorangapp.com/flashcards/28659/Antibody+Structure/#review*

1.7.1 Subtypes of B-cells based on Ig expression

So far only three types of functional Igs; IgM, IgD, IgT/IgZ (IgT) have been identified in teleosts, where the isotype is determined by the constant (C) regions in the heavy chain: Cμ for IgM, Cδ for IgD and Cτ for IgT (93). IgM is evolutionary the most ancient Ig and is produced from early embryotic development in salmonids (94). IgM is the dominating Ig subset in the systemic (spleen and kidney) and peripheral sites (PBL and peritoneal cavity) in teleosts (95-97). IgM exist as a monomer when membrane bound and as a tetramer when secreted in serum (98, 99). IgM therefore has higher avidity (binding strength) when secreted (8 binding sites) compared to membrane bound (2 binding sites) (47, 98). Secreted IgM can bind epitopes closely or far apart due to their flexibility (100, 101). IgM has a role in both the innate (natural antibodies) and adaptive immunity (78). In Atlantic salmon, the concentrations of IgM in plasma vary from 80 – 130 mg/100 mL (98, 102) and their half-life in serum of salmonids is from 1.3 days (103). High affinity (binding of IgM to antigen) and highly polymerized antibodies have a longer half-life than low affinity and low polymerized antibodies (104). IgT

is unique for teleost and is most likely the equivalent of IgA in humans. It is specialized for mucosal immunity in salmonids and has a 100-1000 fold lower concentration in plasma than IgM (105). Most teleosts express only membrane bound IgD, that are co-expressed with IgM on the same cell. Secreted IgD have been detected in rainbow trout, but not yet in Atlantic salmon (98). The role of IgD is however unclear (79). Atlantic salmon possess two Ig heavy chain loci resulting in two IgM sub variants, IgM-A and IgM-B, which both are present serum. However, the biological significance of having two IgM sub variants is incompletely understood. Like IgM, IgD has also been cloned in several teleost species including salmon (106, 107). The structure of membrane bound Ig (mIg) and secreted Ig (sIg or antibodies) are shown in figure 1.6 (99).

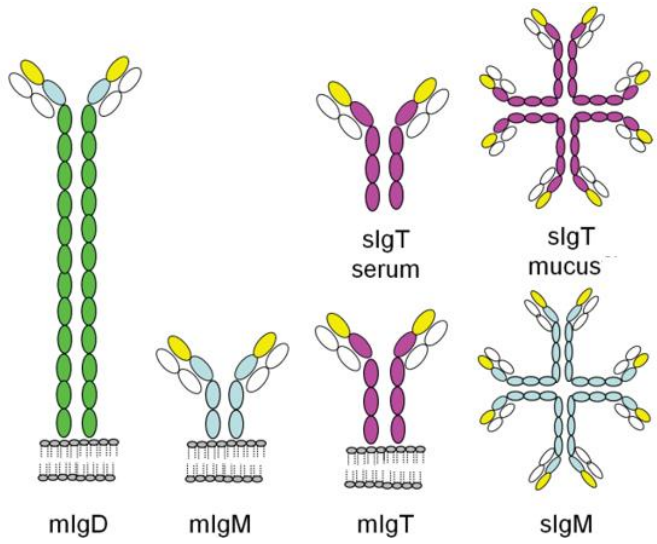

Figure 1.6. Secreted immunoglobulins (sIg or antibodies) and membrane bound immunoglobulins (mIg) in teleosts. *The heavy chain variable regions are shown by yellow ovals, and constant regions are represented by coloured ovals: baby blue, bright pink and bright green, respectively for Cμ, Cτ and Cδ (99).*

1.7.2 Teleost B-cells has similarities with the mammalian B-1 cells

Three independent B-cell progenitors are known to exsist in mammals giving rise to the three B cell phenotypes: B-1a, B-1b and B-2 (conventional B-cells). Conventional B-cells are T-cell dependent and secrete antibodies upon activation (47, 108). B1 cells can be T-cell independent and secrete natural antibodies without contact with antigen (109). Teleost B-cells are long-lived cells that based on the expression of B1 cell marked CD5 (110) and functional characteristics, resemble and is considered as a homologue of the mammalian B-1 cell. These

includes maintained innate functions like expression of PRRs (111), high phagocytic capability (74, 76), as APC where they activate T-cells (87), and secretion of natural antibodies (81). Natural antibodies are polyreactive (unspecific) antibodies with low affinity and can opsonize two or more unrelated antigens (100, 112). Their role is unclear, where they have been found in serum of fish (113), and are often measured as background noise in assays (101). Polyreactive antibodies can self-react to a number of unrelated antigens and cause autoimmunity (100, 114, 115). Polyreactive antibodies are also highly cross-reactive and can bind different antigens (78).

Teleost B-cells are considered to be antibody-making machines secreting IgM both independently (natural antibodies) and dependently of external antigenic stimulation (81, 116). Continuous stimulation by the same external antigen can result in B-cells to become more specific towards antigen (100, 117). The repeated encounters with the same antigen results in point mutations in the variable regions of Ig through the help of activation-induced cytidine deaminase (AID). Thereby increasing the affinity of antibody towards the antigen. This process is termed affinity maturation (47). AID is identified in teleost, but where the affinity maturation process is happening and mechanism around are not well understood teleost (118).

1.7.3 Development and distribution of B-cells

There is a maturation gradient of B-cells in the kidney, where the HK mostly consist of developing pro-/pre-B-cells (rearranging of BCR) in the process of maturation. The naïve B-cells migrate to the sites of activation; the HK, posterior kidney, spleen and periphery and become plasma cells which in turn may circulate back to the HK (63, 119).

Teleost B-cells possess two types of memory: memory B cells and long-lived plasma cells. Memory cells have been through affinity maturation and therefore they react faster and better upon a secondary response (47, 120). Long-lived plasma cells are non-dividing cells that secrete high affinity antibodies. Long-lived plasma cells enter into a compartment (niches) in the HK in teleost (120, 121). HK is a reservoir for long-lived plasma cells where these with the help from other immune cells are kept alive for a long time and maintain persistent antibody secretion (fig. 1.7) (122). A recent study also discovered prolonged local B-cell responses in the peritoneal cavity of Atlantic salmon for up to nine weeks after i.p injection with SAV3, indicating that the peritoneal cavity also could serve as a immunological site by providing a niche for long lived plasma cells (96).

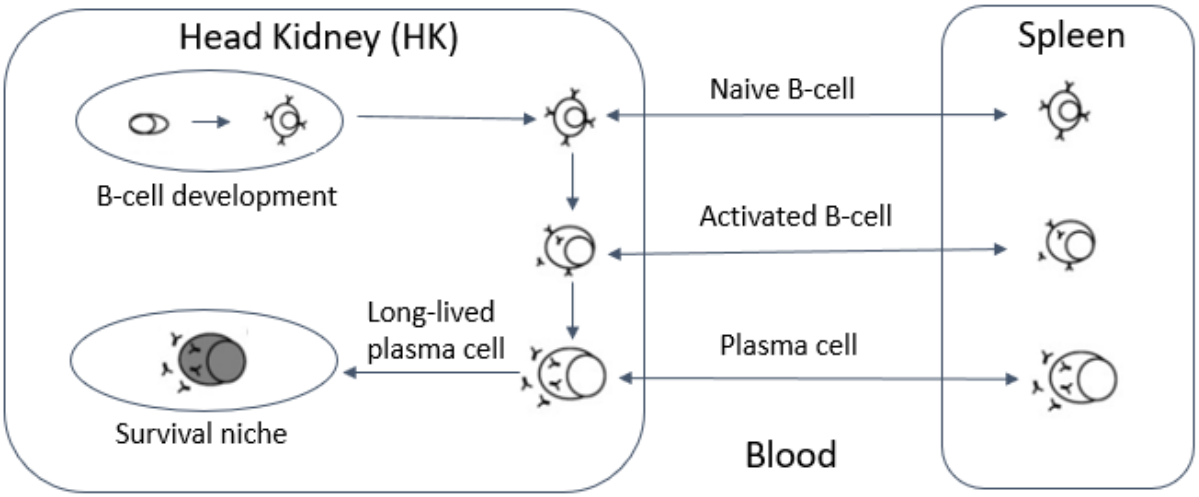

Figure 1.7. B-cell development in HK and migration to/from spleen. *Naïve B-cells are distributed through peripheral tissue through blood. Upon antigen encounter B-cell are activated and mature to plasma cells. Plasma cells can migrate back to the HK where they can enter a survival niche compartment and become long-lived plasma cells (123).*

1.8 Antiviral immune responses

In the aquatic environment, fish are in constantly contact with pathogens, where viruses outnumber other pathogens (124). The first encounter with viruses takes place in the mucosal tissue, where antiviral peptides, enzymes and mucosal antibodies (IgT) fight off the pathogen (63, 125). The immune response against viruses are firstly combated by innate antiviral immune responses (i.e type 1 IFN) and secondly by adaptive immunity (47).

1.8.1 Innate immune responses

In teleosts, recognition of virus occurs through PRRs (i.e. cytoplasmic RIG-1 like receptors, TLR) and result in secretion of type I IFN (126). Type I Interferons (IFN α, β and others) are the most important antiviral defence molecules in the innate immune system. Cells that are infected by virus will start producing and secreting IFN and alarm other nearby cells. The secreted IFNs bind to specific receptors on neighbouring cells and turn on interferon stimulated genes (ISG). This will lead to production of antiviral proteins (Mx protein, ISG15, viperin etc) that inhibit viral replication (degrade genome, inhibit virus assembly etc) (47).

1.8.2 Adaptive immune responses

The adaptive immune system provide immunological memory based on specific antigen recognition and memory development during the second exposure to the antigen. This is the

very basis for vaccination (84, 127). Naïve T-cells are activated by professional APCs and to differentiate into T helper cells (Th) and cytotoxic T lymphocytes (CTL). Th cells produce cytokines (e.g. IFN- γ and IL-2) which are critical in humoral and cellular immune responses against viruses. Th1 stimulates phagocytosis, antigen presentation and increase cytotoxic activity by CTL (47). Teleost lack true germinal centers (GC) and follicles which are important for B-cell proliferation and differentiation to plasma cells in mammals. The lack of follicular structures, point of T-cell and B-cell meeting, makes the role of T-cells towards B-cells uncertain in teleost (47, 80, 81). Activation of teleost B-cell through Th-cells is therefore still a pending question. Melanomacrophage centres observed in the HK and spleen of salmonids contain lymphocytes and macrophages, and might function as the equivalent of GC in mammals. These macrophages might present antigens to B-cells, and thereby take part in B-cell activation and affinity maturation of antibodies (128). The adaptive immune system of teleosts are weaker than that of mammals, as it has limited repertoire of antibodies, a weaker affinity maturation and a slower memory response (80, 81).

1.8.3 The anti-viral effector functions of IgM

The anti-viral effector function of IgM in teleost includes neutralization, agglutination for phagocytosis, opsonization, complement activation and antibody-dependent cell-mediated toxicity (120, 129, 130). Neutralizing antibodies bind specific epitopes (attachment protein) on the virus. The antibodies then prevent the virus from entering the host cell and do harm, thereby neutralizing it (47, 120). Specific IgM with neutralizing properties towards many different viruses have been demonstrated in serum of salmonids (131, 132). Agglutination occurs when IgM link viruses together as a "clump". This prevents the virus to infect more cells. In addition, these "clumps" are more effectively phagocytosed and removed by phagocytic cells (120). IgM can activate the complement system (classical pathway) and contribute to lyse and opsonize pathogens (78, 130, 133). NK-like cells (NCC) are activated by IgM through antibody-dependent cell-mediated cytotoxicity (ADCC) and degranulate and kill infected host cells (129).

Part C:

1.9 Piscine orthoreovirus (PRV)

1.9.1 The PRV particle with its protein components

Piscine orthoreovirus (PRV) is a common virus in salmonid fish, with three recognized subtypes (PRV1-3) which differ in host tropism and are linked to different diseases (134). The virus belongs to the genus *Orthoreovirus* in the family *Reoviridae* (135). PRV is a naked virus with two layers (capsid) of icosahedral structures with a size of approximately 70 – 80 nm in diameter (11, 136). The virus particle contains 10 double stranded RNA (dsRNA) segments; three λ (L), three μ (M) and four σ (S) with a total size of 23,320 nucleotides (nt) that encodes for at least 11 proteins (34). There are 8 proteins assumed to be structural and form the inner and outer capsid, while there are 3 proteins assumed to be non-structural proteins (fig. 1.8) (134).

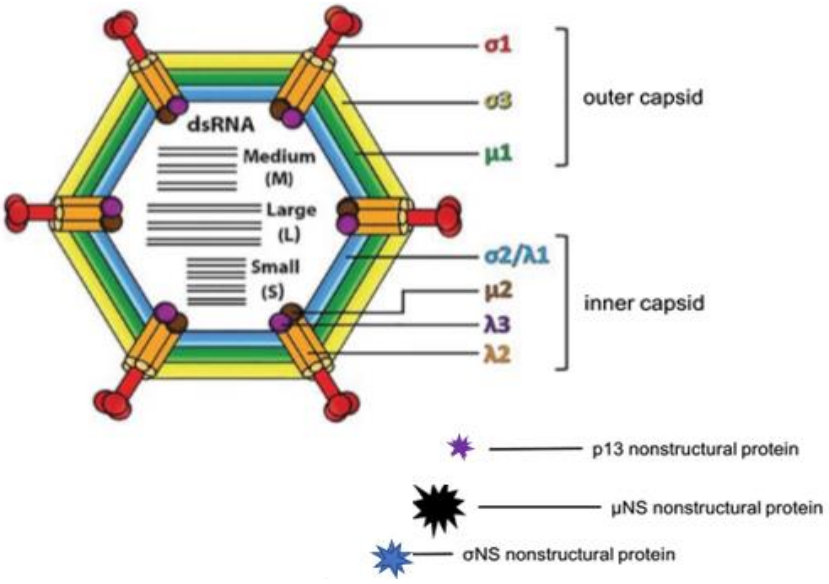

Figure 1.8. Piscine orthoreovirus (PRV). *The structural (outer and inner capsid) and non-structural (ns) proteins of Piscine orthoreovirus (PRV) (137). Illustrates only six of the twelve turrets (138).*

Mammalian orthoreovirus (MRV) is a virus related to PRV that has been extensively studied and is currently used as a model for predicting structural and functional properties of PRV (135). In MRV, the outer capsid consists of σ1, μ1 and σ3 proteins. The σ1 protein is a trimer attachment protein with serotype determination (139, 140). Antibody mediated protection has shown neutralizing functions towards the σ1 protein and an induced production of neutralizing antibodies when σ1 protein is presented (141, 142). The σ1 protein binds to the receptor

junction adhesion molecule-A (Jam-A) in mammalian cells (139, 143), but this is yet to be discovered for PRV. The μ1 protein is the major outside capsid protein, where three subunits of μ1 protein and three subuntis of σ3 protein form a heterohexamer (138). The μ1 and σ3 proteins are proteolytic cleaved in the endosome after viral uptake and are important for viral entry and infectivity. The μ1 will be cleaved into μ1C and a shorter μ1n peptide and release the virus from the endosome to the cytoplasm (144). The viral proteins σ1 has been identified in serum using monoclonal antibodies (132). The cytoplasmic σ3 protein protects and binds dsRNA and prevents translational shutoff. The inner capsid consists of λ1/σ2, λ2, λ3 and μ2 proteins. Here, 12 turrets/spikes are formed by the λ2 and σ1 protein (138). The non-structural proteins in PRV are the σNS, μNS and p13. The σNS and μNS are not part of the viral particle itself but serve a role in replication and host interaction (145). The μNS is the main protein involved in the viral factory formation (globular inclusions) as it recruits the newly replicated PRV proteins for virus assembly (146). Viral factories are produced to shield virus replication from cytoplasmic and endosomal nucleic acid sensing by PRRs (146-148).

1.9.2 *Piscine orthoreovirus* subtype 1 (PRV-1)

PRV-1 has been predominantly identified in salmonid species. Infection by PRV-1 is common for Atlantic salmon, and widely spread geographically as it has been identified in Europe (Norway, Denmark, Faroe Islands, Sweden, Iceland, Ireland, United Kingdom, Germany, France), North America (Canada, United States) and South America (Chile) (149). PRV-1 was first identified in Norway in 2010 through genome sequencing (34) and was connected to HSMI in Atlantic salmon when purified PRV-1 gave illness (11).

Pathogenesis – Atlantic salmon:

PRV-1 infects horizontally (150), and can infect Atlantic salmon though the intestinal wall when given anally. However, the actual route of infection is not well studied, and can be over the gills, orally, fin base e.g. (151).

PRV-1 replicates after a week post-infection (wpc) in erythrocytes. Erythrocytes are seen as the main target cell for PRV where more than 50 percent of all erythrocytes can be infected (136, 152). The spleen has been shown to reflect the levels of PRV infection in blood (136). High plasma viremia was observed and gave massive infection in the erythrocytes. The RNA viral load peaked for around a week in plasma following erythrocytes (153, 154). The viral RNA in serum after decreased and stabilized, establishing a persistent infection. PRV-1

establishes a persistent infection in Atlantic salmon, as the fish fails to eliminate PRV-1 virus (39).

The release mechanism from infected erythrocytes are still unknown for PRV-1 in Atlantic salmon, but anemia is not a clinical sign for PRV-1. However, the presence of µNS in plasma suggest that there is some degree of lysis of erythrocytes. Following the replication of erythrocytes and peak plasmic viremia (154), PRV-1 sheds from faeces to water (151) and can be detected in different cells including cardiomyocytes, macrophages and hepatocytes (154). The infiltration of inflammatory cells in the heart are dominated by CTL, which are the hallmark of HMSI (155, 156). Following infection of cardiomyocytes, PRV-1 can also infect the red skeletal muscle (14). The pathogenesis of PRV-1 in Atlantic salmon is illustrated in fig. 1.9.

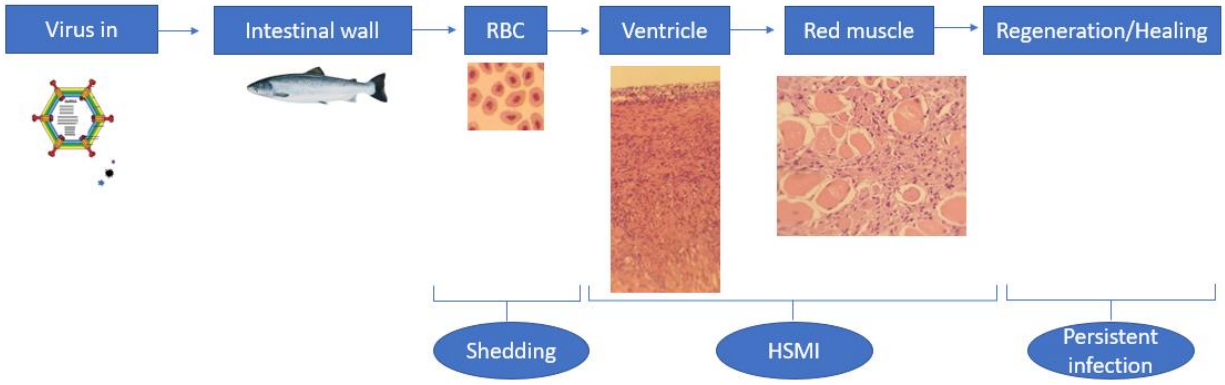

Figure 1.9. Pathogenesis of PRV-1 in Atlantic salmon*. Virus uptake through the intestinal wall, where erythrocytes (RBC) are infected (151). Inflammation in the heart (ventricle) and later the muscle causing HSMI (18). The regeneration/healing process of tissue take place with a persistent infection of PRV-1 (39). Histology pictures taken by Monica Nordberg during pathology course (BIO-2605) at UiT.*

1.9.3 Other subtypes of PRV

There are two additional subtypes of PRV known as PRV-2 and PRV-3 which also infect salmonids but results in different diseases. PRV-2 causes erythrocyte inclusion body syndrome (EIBS) in coho salmon (*Oncorhynchus kisutch*). A disease characterized by acute anemia (157). PRV-3 (also called virus Y or PRVOm) causes HSMI-like lesions (heart inflammation) and anemia in rainbow trout (*Oncorhynchus mykiss*) (158-160). PRV-3 is reported to be successfully cleared in rainbow trout and not move into persistence like PRV-1 in Atlantic salmon (159, 160). For PRV-3, the main host species for infection may be wild brown trout (*Salmo trutta L.*) due to infection prevalence (161). PRV-3 infection has been experimentally

injected into Atlantic salmon to assess the causal relationship between the virus and heart inflammation. During the 10 week long trial, slower transmission, less heart pathology and lower antiviral responses was observed compared to PRV-1 (159). There has never been reports of PRV-3 or PRV-2 infection in Atlantic salmon in the wild, and no trials on PRV-2 infection in Atlantic salmon prior to the one my thesis is based on (162).

1.9.4 The basis of cross-protection of PRV-1

The PRV subtype similarity has to some extent resulted in infection between species with different disease development (163). PRV-3 is closer related to PRV-1 than PRV-2 when comparing amino acids composition and nucleotide composition. PRV-1 and PRV-3 have an amino acid resemblance of 90.5 percent, while PRV-1 and PRV-2 have an amino acid resemblance of 80.3 percent. Cross protection by cross-binding antibodies (PRV specific antibodies) rely on resemblance of amino acid composition between the PRV subtypes. Earlier studies showed that monoclonal antibodies protective against one reovirus also was protective against other reoviruses when targeting the outer capsid proteins (141, 164). The outer capsid protein PRV-1 σ1 has an amino acid resemblance of 81.6 percent and 66.7 percent towards PRV-3 σ1 and PRV-2 σ1, respectively. The PRV-1 outer capsid protein μ1 has an amino acid resemblance of 91.5 percent and 85.1 percent towards PRV-3 μ1 and PRV-2 μ1, respectively. The non-structural protein PRV-3 μNS has an amino acid resemblance of 82.2 percent towards PRV-1 μNS (164).

1.9.5 Immune responses to PRV-1

Innate immunity:

Innate antiviral responses are primarily studied in erythrocytes, as they are the main target cell for infection in salmonids (165). Erythrocytes sense and respond to PRV-1 by the upregulation of innate effector genes resulting in a high IFN-mediated innate antiviral response (59, 166). The interferon stimulated genes (ISG) turned on by IFN in neighbouring cells has shown in mammalian host cells to block MRV (59). However, MRV may continuous its own virus protein production by avoiding the host`s translation block by interacting with PKR (167). This is not the case for PRV, where it is seen a long-lasting production of ISG. The regulation of these genes therefore do not indicate a blockage of IFN signal (168).The observed long lasting secretion of IFN from PRV-1 infected cells may trigger cross-protection against other unrelated

secondary viral infections (IHNV and SAV) as it induces a protective innate antiviral response that might last for several weeks after primary PRV-1 infection (169-171).

T-lymphocytes (T-cells) response:

The recruitment of immune cells parallelly decreases virus levels in heart and suggest that there is a specifically directed immune response towards myocytes. PRV-1 does not directly lyse the cell it infects (136, 172), and the pathogenic potential of PRV-1 can be linked to an adaptive (CTL mediated) immune response by the host (155, 173). Identification of transcript markers of CTL (granzyme A and CD8+) have been found in spleen, HK and heart, indicating a CTL attack on PRV-1 infected cardiomyocytes (174). Furthermore, the heart seems unaffected by PRV-1 infection until the recruitment of immune cells into the epicardium and the compact layers occur. The influx of CTL in the heart (inflammation) is characteristic for HSMI, followed by lysis and necrosis of cells (17, 19, 155).

B-lymphocytes (B-cells) response:

Following a PRV-1 infection, soluble and membrane-bound IgM gene expression was induced in HK (174). Chemokine (CCl19) attracting dendritic cells, T-cells and B-cells with their expressed chemokine receptor CCR7 was found in spleen and HK. In response to PRV-1 infection, both specific antibodies and polyreactive antibodies are induced (116). The increase in polyreactive antibodies is observed as binding to control antigens in immunoassays and was neither observed in rainbow trout infected by PRV-3 nor in Atlantic salmon infected with SAV. Therefore, this seemed like a phenomenon typically induced by PRV-1 in Atlantic salmon (175, 176).

1.9.6 Specific antibody responses against PRV

PRV specific plasma IgM in Atlantic salmon targeting the PRV-1 outer capsids σ1 and μ1c and the viral factory protein μNS, have previously been detected using bead based multiplex immunoassays (116). The PRV-specific antibodies were associated with protective effects, decreased viral loads, decreased epicarditis and the regeneration of the infected heart (116, 132).

1.10 Aims of the study

1.10.1 Main goal

This master thesis is a part of the research project VivaAct, which focus on characterizing immune responses triggered by attenuated and inactivated viruses in Atlantic salmon (2018-2021). The main goal of this thesis is to find out if a primary infection with PRV-2 and PRV-3 can result in specific antibodies (i.e., an IgM response) against PRV in Atlantic salmon. This is done through 5 sub-goals.

1.10.2 Sub-goals

Sub-goal 1:

Measure the PRV specific plasma immune response (IgM) in Atlantic salmon over an 18-week period post infected with PRV-1.

Sub-goal 2:

Measure the PRV specific plasma immune response (IgM) in Atlantic salmon over a 10-week period post immunization with PRV-2, PRV-3, and inactivated PRV-1.

Sub-goal 3:

Measure the PRV specific plasma immune response (IgM) after introduction of PRV-1 shedders to Atlantic salmon immunization with PRV-2, PRV-3, and inactivated PRV-1.

Sub-goal 4:

Compare the PRV specific plasma immune response (IgM) in Atlantic salmon over an 18-week period post immunization with PRV-2, PRV-3 an inactivated PRV-1, using PRV-1 infection as a positive control and Mock as negative control.

Sub-goal 5:

Perform PRV specific antibody analyses on two different Bio-Plex 200 machines.

2.0 Material & Methods

2.1 Description of vaccine trial

The vaccine trial in Atlantic salmon was planned as part of the RCN-funded ViVaAct project, aiming to compare the effect of live, attenuated vaccines with inactivated vaccines against PRV and SAV. The trial lasted for 18 weeks and was divided into two periods. Period I was defined by the 10 first weeks of the trial. Here, the focus was on the immune response of the inactivated PRV-1 vaccine (InPRV-1) and the two viruses acting as attenuated "live" vaccines: PRV-2 and PRV-3. Period II was defined by the last 8 weeks of the trial and focused on the effect of the vaccines on a secondary infection (fig. 2.1).

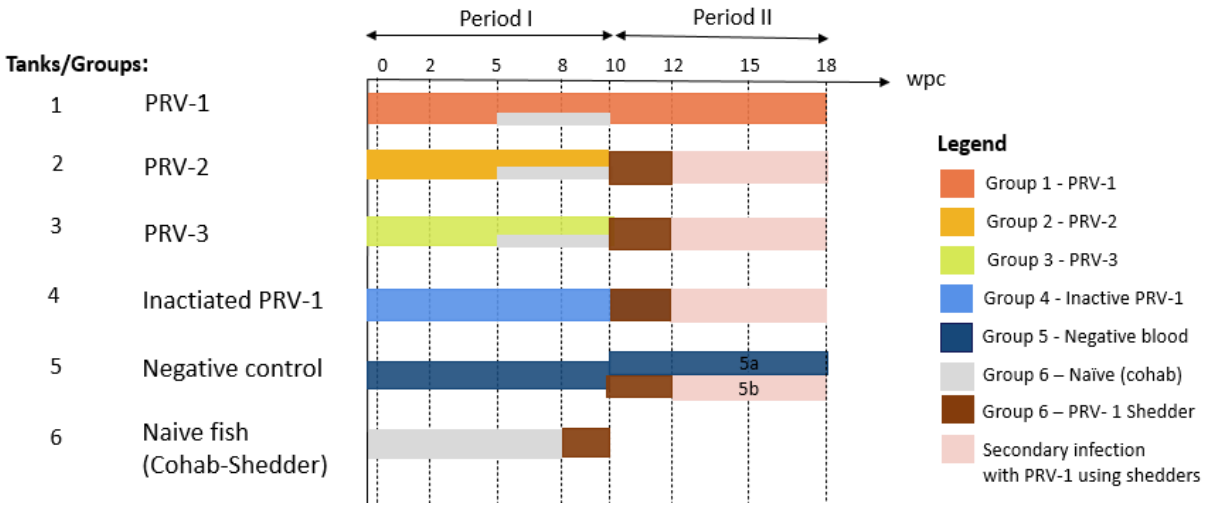

Figure 2.1. Timeline of the ViVaAct project. *The trial lasted 18 weeks where there were six different tanks/groups; PRV-1, PRV-2, PRV-3, inactivated PRV-1, Negative controls and Naïve fish (later used as cohabs/shedders). Shedders were added 10 weeks post challenge (wpc) and split the experiment in two periods. Period I was the first 10 weeks of the trial (0-10 wpc) before PRV-1 shedders were introduced. Period II was the last 8 weeks of the trial (10-18 wpc) after PRV-1 shedders were introduced.*

Atlantic salmon were placed in six different 50 L freshwater tanks (groups), where group 1, 2 and 3 contained 75 fish injected with three different PRV variants; PRV-1, PRV-2, PRV-3, respectively. PRV-1 was isolated and prepared from blood cells of infected Atlantic salmon from a previous trial (11), and originated from an outbreak of HSMI in mid Norway in 2012. PRV-2 was isolated from spleen tissue of coho salmon transported from Japan (157). PRV-3 was isolated from blood cells originating from infected rainbow trout sampled during an outbreak in Norway in 2014 (158). Tank 4 contained 75 fish injected with formalin inactivated PRV-1 with adjuvants prepared by PHARMAQ/ ZOETIS. The adjuvant was a water-in-oil

formulation (60). Tank 5 contained 125 uninfected fish (negative controls) with injected blood without PRV infection (negative blood/mock), and tank 6 stored 190 naïve fish later used as cohabitants or shedders. At 5 wpc, 15 naïve fish were introduced to each of the groups 1, 2 and 3 to check if the viruses were transmitted to cohabitants. Three and five weeks after addition (8 and 10 wpc), cohabitants were sampled/examinated for virus and at 10 wpc PRV-1 infected shedder fish were introduced to group 2, 3, 4 and 5b which was separated from group 5a (mock) to check immunity towards PRV-1 and protection from HSMI. To differentiate between immunized and introduced fish (cohab/shedder), the introduced fish were tattooed with a blue dye on the ventral side.

Atlantic salmon had an average start weight of 45 g (0 wpc) and an average end weight of 194 g (18 wpc). Fish were fed Nutra Olympic (Skretting) and had optimal environmental conditions during the trial period with temperature of 10 degrees[2], oxygen varying between 80-100 percent and 24 hours of light. There was no mortality in any groups during the trial. The trial was performed at Tromsø Aquaculture Research Station in Kårvika.

For my master thesis, antibodies in blood plasma from all groups were analysed in week 0, 2, 5, 8, 10, 12, 15 and 18. I participated in the last 11 weeks of the trial and joined sampling at 8, 10 and 15 wpc at Tromsø Aquaculture Research Station in Kårvika. Antibody analysis from week 0-15 was conducted using the Bio-Plex 200 machine at the Norwegian University of Life Sciences (NMBU) and the Veterinary Institute (VI) in Oslo, together with PhD student Lena H. Teige (NMBU) and laboratory engineer Karen Bækken Soleim (NVI), respectively, while samples from week 18 were analysed in my absence by Karen Bækken Soleim.

[2] If temperature fell under with 0.5 degrees or above with 0.5 degrees, measures were initiated (procedure at Tromsø Aquaculture Research Station).

2.2 Immunization and sampling – Tromsø Aquaculture Research Station

Blood samples were taken from 8 fish at 0 wpc (end of the acclimation period) before the immunization started. The fish in groups 1-4 were injected with 0.2 mL of immunization material, and group 5 with control material without virus. Blood sampling from i.p injected fish (groups 1-5) was performed week 2, 5, 8, 10, 12, 15 and 18, where 8 fish from each group were sampled. In addition, blood was sampled from 6 cohabitant fish (transmission controls) at 8 and 10 wpc. Each plasma sample were given a code of three numbers in excel. The first number defined what study week the plasma was sampled, the second number explained what experimental group the plasma sample was collected from, and the third number was the identification number of the fish (sec. 6.1 Appendix).

2.2.1 Sampling

The Aquaculture Research Station was a 30 minutes' drive from Tromsø airport (28,9 km) (fig. 2.2). At the Research station, infected and non-infected fish were divided into two separate rooms to avoid contamination of tanks and samples. For period I (0 – 10 wpc), group 1, 2 and 3 (infected with replicating virus) was in an infection room, while group 4 and 5 was in a non-infection room. Group 6 however, was moved into the infection room in week 8 and infected with PRV-1 to be used as shedder fish. In week 10, both group 4 and half of group 5 (5b) was moved to the infected room, and shedders were introduced in these tanks, while the other half of group 5 (5a) was left alone in the non-infected room as a control group.

To avoid cross infection of different viruses between groups, sampling was first completed on group 5, followed by group 4, 2, 3 and 1. Therefore, groups in the non-infected room were sampled before groups in the infected room. To avoid contamination between groups and samples, gloves and equipment (scissors, tweezer, scalpel, table paper) was changed and the work bench was sterilized after each group. In addition, scalpel blades and needles were changed between each fish. After week 10, all groups (except 5a) were infected with shedders from PRV-1. It was therefore not as crucial to change and sterilize everything between infected tanks in period II (10-18 wpc) as it was in period I (0 – 10 wpc).

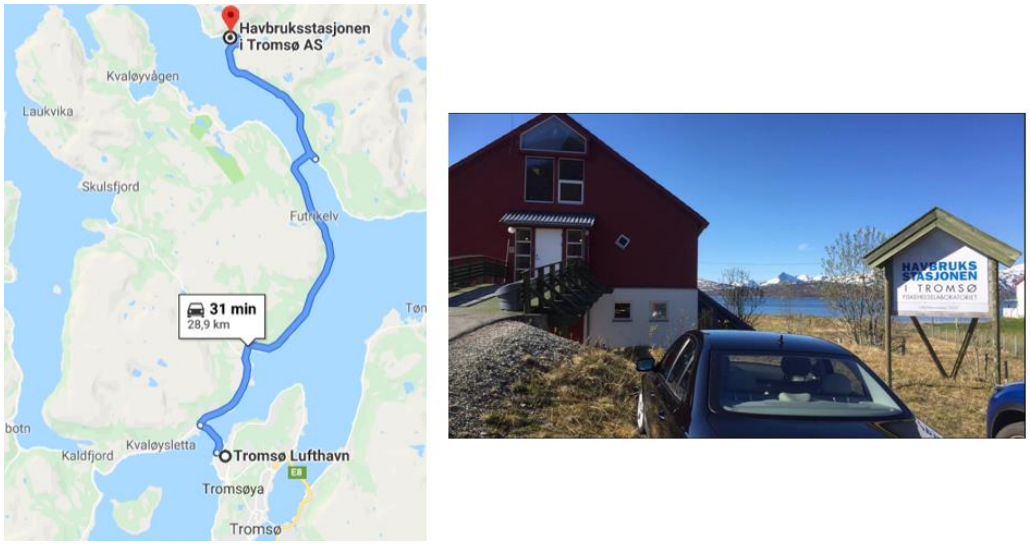

Figure 2.2. The Aquaculture Research Station in Tromsø, Kårvika*. To the left, road map from Tromsø airport to the sampling spot at Tromsø Aquaculture Research Station. Retrieved from google maps. To the right, Tromsø Aquaculture Research Station, site of trial.*

Upon sampling, the Atlantic salmon was netted into a bucked containing an overdose of benzocaine. Blood samples were drawn immediately post-mortem from the caudal vein (*aorta*/*vena caudalis)* (fig. 2.3). Blood was sampled into Li-heparin vacutainer tubes (4mL) and stored on ice. The blood samples were centrifuged for 10 minutes at 3000g and 4 degrees on Multifuge Heraeus 1 s-r (Id-Nr: 20057821-a) later the same day. The plasma samples were pipetted into a micro tube (700 µL) and temporarily stored on minus 20 degrees at the Artic University of Norway (UiT). The day after, the plasma samples were packed with cooler elements when transferred to NVI in Oslo. Here, the plasma samples were stored on minus 80 degrees until further analysis.

Figure 2.3. Blood sampling at Aquaculture Research Station in Tromsø, Kårvika

Blood sampling of Atlantic salmon at 8 wpc.

2.3 Preparation of bead-based antibody assay

2.3.1 The principal of Bio-Plex

Bio-Plex (Luminex) assay is a sensitive bead based multiplex assay that enables results in a shorter time with less work, and precise results with less sample material, compared to other analytical techniques (i.e ELISA). The beads are magnetic and coloured internally with ten different colours of red and infrared fluorescence dye, which in combination makes up 100 different beads (fig. 2.4). Each bead type can be coated with a protein (sec. 2.3.2). The beads can be distinguished from the other beads due to its different red and infrared fluorescens dye. Different proteins can therefore also be combined in the same analytical sample well, so called multiplexing (177).

Plasma contains a pool of different polyclonal antibodies (178). Polyclonal antibodies are produced by different clones of plasma B-cells targeting the same antigen, but different epitopes (179). During analysis, plasma was heat treated to decrease binding of polyreactive antibodies before added to coated beads (116). The primary Mouse Anti-Salmonid IgH monoclonal antibody (Cedarlane/Nordic BioSite, cat. CLF004, size 250 µg) was added to plasma antibodies attached to beads (fig. 2.7). Monoclonal antibody is produced by identical B-cells that are cloned from a single parent cell. To produce monoclonal antibodies, mouse is injected with a specific antigen (salmonid antibodies), multiple times. Spleen cells are isolated and fused with myeloma cells. The hybrid cell (hybridoma) are selected and cultured to produce identical monoclonal antibodies against heavy chain of salmonid antibody (179). Monoclonal antibodies are specific and react only with its introduced epitope (in this case salmonid IgH) and not with any other antigen (100). The secondary biotinylated Goat Anti-Mouse-IgG2a polyclonal antibody (SouthernBiotech, cat. 1080-09, concentration of 0.5 µg/µL) was added after the primary antibody (fig. 2.7). Polyclonal antibodies are produced by goat being injected with a specific antigen (Anti-Mouse-IgG2a). A mixture of antibodies is produced from different clones of plasma B-cells, where they target the same antigen but different epitopes. These polyclonal antibodies are harvested and isolated from serum of goat (179). Streptavidin-PE conjugate is added to the biotinylated secondary antibody (fig. 2.7). Streptavidin bind biotin and give a light signal (PE) which can be detected by the Bio-Plex 200 machine as mean fluorescence intensity (MFI). MFI signal is therefore correlated to plasma antibodies bound to beads (177).

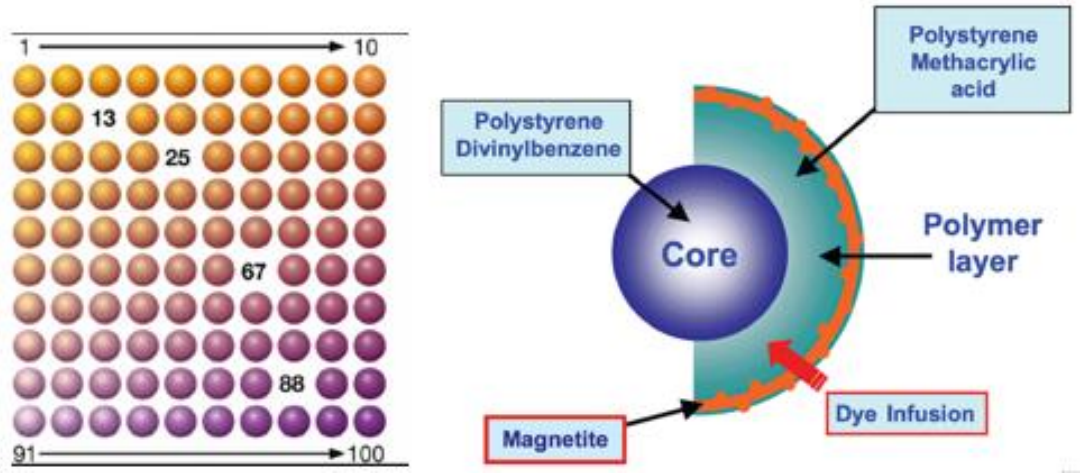

Figure 2.4. Magnetic beads used for multiplex assay. *To the left, the bead region/gate of 100 different coloured bio-plex beads with their bead number 1-100. The different beads can be in the same well and target up to 100 different analytes at the same time. To the right, beads during the staining process. The dye contains a solvent that allow the beads to swell and coat the polymer layer of the bead. The removal of this solvent trap the dye inside the bead and results in many uniquely coloured beads (177).*

The beads are pushed through a detection chamber, which emits red (635 nm) and green (532 nm) laser, where the red classification laser identifies the bead colour, and thereby the gate/region of the beads (fig. 2.4). The green reporter laser identifies the intensity of red fluorescence reporter signal (streptavidin-PE binding to biotin) (fig. 2.5). The maximum fluorescence of the green laser that can be obtained is 25000 mean fluorescence intensity (MFI). At 25000 MFI, all proteins (antigens) have analytes (antibodies) attached (177).

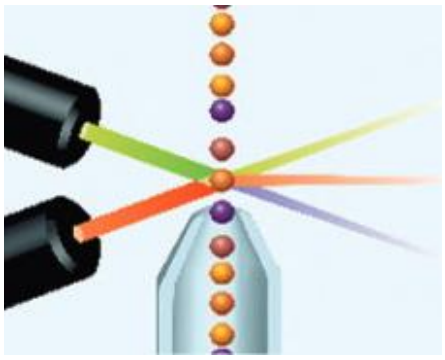

Figure 2.5. Beads pushed though the detection chamber*. Beads are identified by the red classification laser and the concentration of analyte (antibodies) to each bead are measured by the green reporter laser (177).*

Bio-Rad recommended bead count to be set to 50 beads in each region. This would ensure that at least 50 beads per region would be acquired before next well could be analysed. A time limit to each well was set to ensure that the reading did not continue for an extended length of time (0-200 sec). The flow of beads, the bead count, the bead region, and the platform temperature were monitored. If the system detected a problem in any of these areas, it triggered sample errors (table 2.1) (180).

Table 2.1. Different errors, error triggers and error causes during Bio-Plex 200. *Error 1, 2 and 4 was triggered during Bio-Plex run of plasma samples (180).*

Error:	*Error triggered*	*Possible cause:*
1. Low bead number	*If there are less than 25 % of acquired beads per bead population*	*Too few beads in the assay; buffer volume in well is too low; plate was not shaken properly before analysis; microbubbles in the cuvette.*
2. Aggregated beads	*Above 50 percent of aggregation in well*	*Bead clumping in the assay; sheath fluid is empty; waste reservoir is overfull*
3. Classify efficiency		*Microbubbles in the cuvette; beads have become photobleached; percentage of beads outside the selected bead region is too high.*
4. Region selection	*If there are less than 20 beads counted for each bead population*	*Incorrect beads were selected in the Protocol or assay; too few beads are present in the assay*
5. Platform temperature		*Platform temperature has fluctuated ±2ºC during the reading.*

2.3.2 The principle of bead coating

The surface of beads is covered with carboxyl groups, which offers multiple attachment sites for proteins. The attachment of proteins to the beads is called "coupling" reaction. The carboxyl groups on the beads are reactive and it is therefore easy to couple a desired protein to the bead. The COOH groups exposed on the beads are activated by a simple two step carbodiimide reaction, where N-hydroxysulfosuccinimide (S-NHS) reacts with N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDAC) to form a S-NHS-ester intermediate (active ester). This active ester can react with the primary amine group (NH_2) of

a protein and form a permanent covalent attachment between the protein and bead (fig. 2.6) (181).

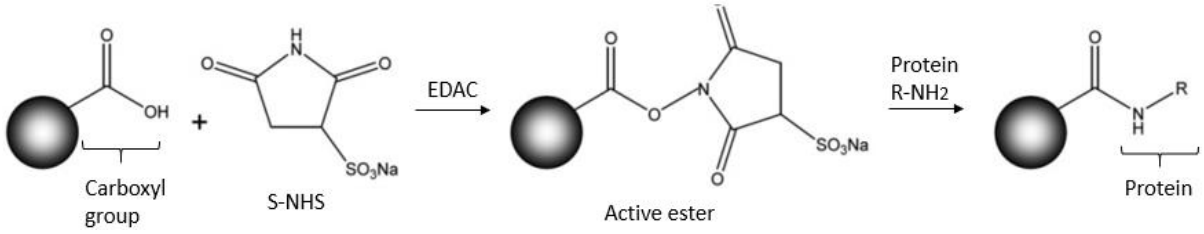

Figure 2.6. Coating of protein (antigen) to beads. *Coupling reaction where the carboxyl group is activated by S-NHS and EDAC to form an active ester which bind the primary amino group (NH_2) in proteins. Retrieved from Bio-Rad`s instructions manual.*

Antibodies bind specific epitopes on the protein, and it is therefore important that the coated protein mimic the structure of the protein as it is presented in the fish. The higher similarity between the protein on the bead and the protein presented in the fish, the higher the chance that the antibodies bind to the protein. How the proteins are coated to the bead are therefore of importance for antibody detection in plasma of Atlantic salmon. The manufacturers for each of the proteins used in this master thesis are listed in table.2.2.

Recombinant PRV-1 σ1 and PRV-1 μ1c proteins were produced in *Escherichia coli* (E.coli) (172). In 2019, lipid modified PRV-1 σ1 (σ1-LM) was discovered to be a sensitive and reliable antigen for detection of anti-PRV-1 antibodies in plasma of Atlantic salmon (116). The lipid modification of the N-terminal of PRV-1 σ1 increased specific detection of anti-PRV IgM, whereas PRV-1 σ1 failed to bind antibodies without the lipid modification. This was proposed to be related to the way the PRV-1 σ1-LM was coated on the beads, where the lipid either made the protein more available for the antibodies to bind or stabilized its optimal structure (116, 132). The viral PRV-1 μ1C and PRV-1 μNS proteins have also been used to detect antibodies infected with PRV-1 in Atlantic salmon (132), but the bead coating of these antigens to beads have not yet been identified to only bind specific antibodies (polyclonal) towards PRV-1 as PRV-1 σ1-LM (116). In this trial, PRV-1 μNS was not used, as the protein was not available at the time plasma samples were analysed. The PRV-3 proteins σ1 and μNS were tried out for the first time to check if they could detect specific antibodies from PRV-3 infected fish. In addition, three control proteins from the infectious salmon anemia virus (ISAV) were analysed with plasma samples. ISAV FP-LM worked as a control protein for PRV-1 σ1-LM as they were both outer capsid proteins that were lipid modified, while unmodified ISAV FP worked as a

control protein for PRV-1 µ1C, as both are endosome fusion proteins. The lipid modified ICP11 (ICP11-LM), a protein from white spot syndrome virus that infect shrimp was added as an additional control protein that salmon could definitely not have been exposed to earlier. ICP11 is a non-structural protein that acts as a DNA mimic (182).

Table 2.2 Overview of the manufacturer of the antigens and protein concentration.

Manufactured by	Antigen w. concentration
Kumar Subramani	*ILA FP (0.046 mg/ml)*
Kumar Subramani	*ILA FP-LM (0.054 mg/ml)*
Kumar Subramani	*ICP11-LM (0.2 mg/ml)*
Øystein Wessel	*PRV-1 µ1c (0.62 mg/ml)*
Kumar Subramani	*PRV-1 σ1-LM (0.12 mg/ml)*
Ingvild Berg Nyman	*PRV-3 σ1 (0.4 mg/ml)*
Ingvild Berg Nyman	*PRV-3 µNS (0.4 mg/ml)*

2.3.3 Bead coating

The coupling reaction was performed according to the Bio-Rad`s instructions manual; Bio-Plex Pro™ Magnetic COOH Beads Amine Coupling Kit (BIO-RAD, cat.171-406001). The coupling kit contained wash buffer, bead activation buffer, phosphate buffered saline (PBS buffer), blocking buffer and storage buffer.

Antigens used for bead coating:

- Two PRV-1 proteins (σ1-LM and µ1C).
- Two PRV-3 proteins (σ1 and µNS).
- Three control proteins (ISAV FP, ISAV FP-LM and ICP11-LM).

New Eppendorf tubes (bead tubes) were marked with bead number and protein. The COOH beads (beads) with a stock concentration of 1.25 x 10^7 beads/mL were vortexed for 30 seconds and stored into a floating rack added cold water and sonicated by sound waves for 15 seconds. Beads were added to bead tubes according to the amount of protein available at this time point. How many beads, how much protein in what volume during coating, and the ratio of protein to beads can be seen in table 2.3.

After beads were added, step 1-11 was completed:

1. Tubes were placed on a plastic rack onto a magnetic separator, Dynamag^{TM} -2 (Thermo Fisher Scientific, cat. 12321D) for 30 – 60 seconds to allow the beads to attach to the magnet.
2. The supernatant was pipetted away carefully to avoid removal of beads. Pipette tip was changed between each bead tube.
3. The plastic rack was lifted away from the Dynamag^{TM} – 2.
4. Each bead tube was added 100 µL wash buffer and vortexed for 30 sec. Step 1-3 was repeated.
5. Bead tube was added 80 µL of bead activation buffer and vortexed for 30 seconds. The bead tubes were put in a drawer away from light.
6. 10 mg of N-hydroxysulfosuccinimide (S-NHS) and 10 mg of N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDAC) was weighed into two new Eppendorf tubes. 200µl bead activation buffer was added to the S-NHS and EDAC tubes, and both tubes were vortexed for 30 seconds.
7. Bead tubes were added 10 µL S-NHS and vortexed for 30 seconds, followed by 10 µL of EDAC and vortexed for 30 seconds. To avoid light, the bead tubes were wrapped in aluminium foil and put in the HulaMixer – Sample Mixer (Thermo Fisher Scientific, cat. 15920D) at room temperature for 20 minutes.
8. 150 µl of PBS buffer (pH 7.4) was added to bead tubes followed by 10 seconds vortex. Step 1–3 was repeated.
9. Bead tubes were added 150 µL PBS buffer (pH 7.4) and vortexed for 10 seconds. Step 1-3 was repeated.
10. Bead tubes were added 100 µL PBS buffer (pH 7.4) and vortexed for 30 seconds.
11. Finally, protein and PBS buffer were added to beads (Appendix sec. 6.3). The instruction manual noted that one coupling reaction equalled 1.25 x 10^6 beads and required 5-12 µg protein.

Here, 12 µg protein were added for the 1st coupling reaction performed (table 2.3). Different coupling scale reactions were completed depending on amount of protein available at this time point. A x1 coupling reaction was completed for bead 27 and 28. A three-fold coupling reaction (x3) was completed for bead 29, 34, 44, 54 and 64. The protein coating had a concentration of

24 µg protein/mL, while the protein/bead – ratio in coating tube was 9.6 protein per million beads. Volume of protein and PBS buffer (pH 7.4) added is calculated in Appendix sec. 6.3.

Table 2.3. 1st coating of beads used in Bio-Plex analysis. *Beads and proteins added to bead tubes with final ratio. A single (x1) coupling reaction equalled 1.25 x 10^6 beads with 12 µg protein in a volume adjusted to 500 µL by PBS buffer (pH 7.4), according to Bio-Rad`s instructions manual.*

Protein (Bead no.)	ISAV FP (27)	ISAV FP-LM (28)	ICP11-LM (29)	PRV-1 µ1C (34)	PRV-1 σ1-LM (44)	PRV-3 σ1 (54)	PRV-3 µNS (64)
Coupling reaction:	x1	x1	x3	x3	x3	x3	x3
Beads added (number)	*1.25 x10^6*	*1.25 x10^6*	*3.75 x10^6*	*3.75 x10^6*	*3.75 x10^6*	*3.75 x10^6*	*3.75 x10^6*
Protein added (µg)	*12*	*12*	*36*	*36*	*36*	*36*	*36*
Final volume (mL)	*0.50*	*0.50*	*1.50*	*1.50*	*1.50*	*1.50*	*1.50*
Final bead concentration (beads/mL)	*2.5 x10^6*	*2.5 x10^6*	*2.5 x10^6*	*2.5 x10^6*	*2.5 x10^6*	*2.5 x10^6*	*2.5 x10^6*
Final protein concentration (µg/mL)	*24*	*24*	*24*	*24*	*24*	*24*	*24*
Ratio (µg protein/beads)	*9.6 µg/ million beads*	*9.6 µg/ million beads*	*9.6 µg/ million beads*	*9.6 µg/ million beads*	*9.6 µg/ million beads*	*9.6 µg/ million beads*	*9.6 µg/ million beads*

There was also a second coating of beads performed, not used in further analysis. Here, different concentrations of protein were added when coating (table 2.4). Depending on amount of protein available at this time point. A x0.5 coupling reaction was completed for bead 27. A three-time coupling reaction (x3) was completed for bead 29, 34 and 44. Bead 27 and 44 had different protein/bead – ratio during coating. Volume of protein and PBS buffer (pH 7.4) calculated in Appendix sec. 6.3.

Table 2.4. 2nd coating of beads used in Bio-Plex analysis. *Beads and proteins added to bead tubes with final ratio. A single (x1) coupling reaction equalled 1.25 x 10^6 beads with 12 µg protein in a volume adjusted to 500 µL by PBS buffer (pH 7.4), according to Bio-Rad`s instructions manual.*

Protein (Bead no.)	*ISAV FP (27)*	*ICP11-LM (29)*	*PRV-1 µ1C (34)*	*PRV-1 σ1-LM (44)*
Coupling reaction:	*x0.5*	*x3*	*x3*	*x3*
Beads added (number)	*6.25 x10^5*	*3.75 x10^6*	*3.75 x10^6*	*3.75 x10^6*
Protein added (µg)	*5.3*	*36*	*36*	*27.36*
Final volume (mL)	*0.25*	*1.50*	*1.50*	*1.50*
Final bead concentration (beads/mL)	*2.5 x10^6*	*2.5 x10^6*	*2.5 x10^6*	*2.5 x10^6*
Final protein concentration (µg/mL)	*21.2*	*24*	*24*	*18.2*
Ratio (µg protein/beads)	*8.48* µg / million beads	*9.6* µg/ *million beads*	*9.6* µg/ *million beads*	*7.30* µg/ *million beads*

The bead tubes were vortexed, packed in aluminium foil and incubated for 2 hours at room temperature in the HulaMixer. After 2 hours, the bead tubes were unwrapped from the foil and step 1-3 was repeated. The bead tubes were then added 500 µL PBS buffer (pH 7.4) and vortexed for 30 seconds before step 1-3 was repeated. The bead tubes were added 250 µL Blocking buffer and vortexed for 15 seconds. The tubes were packed in aluminium foil and incubated in the HulaMixer at room temperature for 30 minutes. After 30 minutes, bead tubes were unwrapped from the foil and step 1-3 was repeated. The bead tubes were added 500 µL Storage buffer and vortexed for 20 seconds before step 1-3 was repeated. Finally, bead tubes were added 150 µL Storage buffer and placed into a new black Eppendorf tube (black tube).
The bead concentration and aggregation of beads were checked. A 10 – fold dilution of the coated beads was made, where 18 µL Storage buffers and and 2 µL coated beads were added to a microtiter plate, respectively. Before pipetting beads, the black tube was vortexed and mixed by pipetting. The storage buffer and coated beads were mixed by pipetting and 10 µL of the diluted sample was taken onto a countess chamber and put in Countess II FL, invitrogen (Thermo Fisher Scientific, cat. AMQAF1000). The concentrations of coated beads are listed in sec. 6.3 Appendix table 2.17. How many beads at the start and at the end of the coating together with bead loss is found in results sec. 3.1.

The black tubes were stored in the fridge at 4 degrees. The proteins, S-NHS and EDAC solutions were stored at minus 20 degrees, while the uncoated beads and kit were stored in the fridge at 4 degrees.

2.3.4 Flow buffer preparation

Flow buffer was made from 50 mL of 10x Flow buffer added to an autoclaved bottle of 450 mL distilled H_2O. 8.3 mL of 30 % bovine serum albumin (BSA) was added to the solution and gave a final concentration of 0.5 % BSA. The lid was tightened, and the solution was mixed by shaking the bottle.

2.3.5 Plasma preparation

A 10-fold dilution of plasma samples was prepared at NVI. The plasma was taken from minus 80 degrees onto ice for thawing. PBS buffer (pH 7.2) and a microtiter plate was placed on ice to ensure cold environment for the samples. 20 µL of plasma was pipetted into the microtiter plate according to the microtiter plate setup (sec. 6.1 Appendix). When all wells were filled with plasma, 180 µL PBS buffer (pH 7.2) was pipetted onto the microtiter plate. Air bubbles and volume in the pipette tips were checked to ensure that the same volume was pipetted each time. The diluted samples were resuspended three times to homogenize the solution and stored at minus 20 degrees.

2.4 Execution of bead-based antibody assay

In this section, the steps involved in the bead-based antibody analysis will be explained. An overview of the procedure is shown in fig. 2.7. The antibody assay was analysed on a black flat bottom 96 well plate (Bio-Rad), shielding the analytes from light.

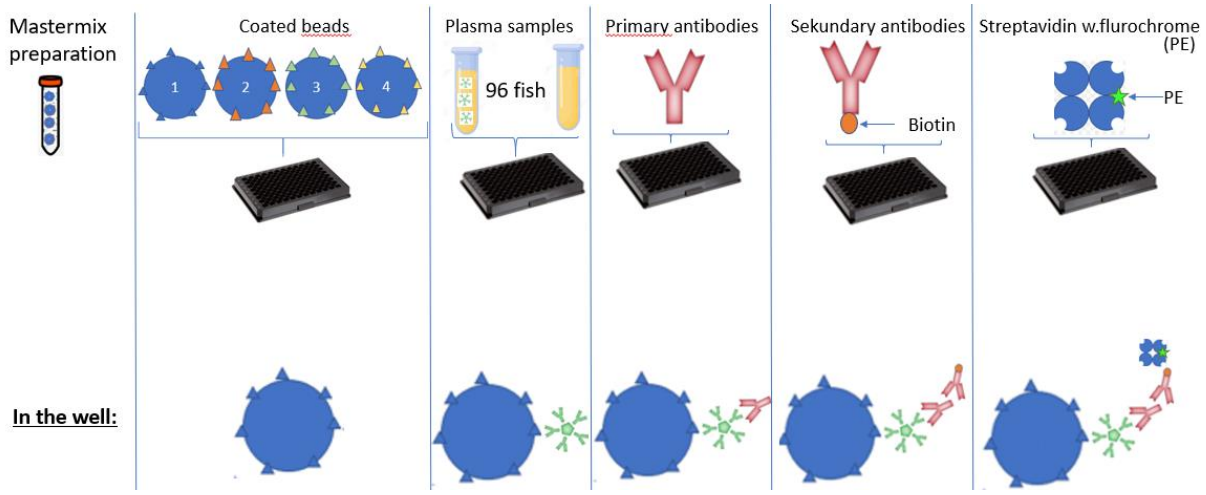

Figure 2.7. The lab preparation of the 96-well plate for Bio-Plex analyses.

Seven 96-well plates were analysed on Bio-Plex. The un-used wells in the plate were covered by tape. The 96-well plate overviews of plasma samples analysed on Bio-Plex can be found in Appendix sec. 6.2.

2.4.1 Mastermix added to the 96-well plate.

Beads were vortexed and pipetted into a new 15 mL tube together with prepared flow buffer. Each sample (well) contained 2500 beads of each bead number and the mastermix volume was 50 µL and had a concentration of 50 beads of each type/µL. Calculations can be seen in sec. 6.4 in Appendix.

Washing beads:

200 µL flow buffer was added to each well, followed by 50 µL of bead mastermix (2500 beads). The mastermix tube was vortexed for 30 seconds and pipetted several times before and after transfer to the plate to ensure that all beads left the pipette tip. A black lid was placed on the plate to protect the beads from light and the plate was incubated on a Heidolph™ Titramax Vibrating Platform Shaker (Fisher scientific, cat. 13-889-871) at room temperature at 750 rpm

for 30 seconds. The plate was placed on a magnet for 1 minute. The magnetic beads were attracted to the magnet and the supernatant could therefore be emptied in the sink by force. The plate on the magnet was tapped three times on paper before the plate was discharged.

2.4.2 Plasma samples

Heat treatment of plasma samples:

The premade 10-fold diluted plasma samples in the microtiter plates were thawed at room temperature. The plasma samples were heat treated on a Heidolph flat hood incubator 1000 (Fisher scientific, cat. 544-12200-00-4) for 20 min at 48 degrees and 150 rpm to decrease binding of unspecific components to the beads (116). Heat treatment was only done once and not repeated for the microtiter plates with 10-fold diluted plasma.

100-fold dilution of plasma samples:

A 100-fold dilution was prepared from the premade 10-fold diluted plasma samples according to the 96-well plate overview in sec. 6.2 in Appendix. Flow buffer was emptied into a disposable box so a multi pipette could be used when pipetting 67.5 µL of flow buffer to a new microtiter plate. 7.5 µL of plasma was then added into each of the wells with flow buffer to a total of 75 µl.

Plasma samples added to the 96-well plate:

50 µL of the 100-fold diluted plasma samples was added to each well of the 96-well plate using a multi pipette. The pipette tips were changed for each plasma sample. A black lid was placed on top of the plate and the plate was incubated on a Heidolph™ Titramax Vibrating Platform Shaker at room temperature at 750 rpm for 30 minutes. The plate was washed three times (step 1-4 below repeated three times).

1. 200 µL flow buffer was added to each well.
2. The plate with lid was incubated on a Heidolph™ Titramax Vibrating Platform shaker at room temperature at 750 rpm for 30 seconds.
3. Plate was placed on a magnet for 1 minute.
4. The supernatant was emptied in the sink and the plate was tapped three times on clean paper. The plate was removed from the magnet.

2.4.3 Primary antibody, secondary antibody, and streptavidin

The primary Mouse Anti-Salmonid IgH monoclonal antibody (Cedarlane/Nordic BioSite, cat. CLF004, size 250 µg) was diluted 1:400 in flow buffer to a concentration of 0.62 µg/µL. The secondary biotinylated Goat Anti-Mouse-IgG2a polyclonal antibody (SouthernBiotech, cat. 1080-09, concentration of 0.5 µg/µL) was diluted 1:1000 in flow buffer to a concentration of 0.5 ng/µL. The reporter fluorochrome, streptavidin-PE conjugate (Invitrogen) was diluted 1:50 in flow buffer to 20 µg/mL. The volume calculated for all 96-well plates can be seen in Appendix sec. 6.5.

Flow buffer was added to three 15 mL tubes followed by primary antibody, secondary antibody and streptavidin in separate tubes. Tubes were vortexed for 30 sec and put in a drawer away from light. A volume of 50 µL of diluted primary antibody, secondary antibody and streptavidin was added to wells with three repetitive washing steps (step 1-4 below) between each step. For each step, a black lid was placed on top of the plate and it was put on Heidolph™ Titramax Vibrating Platform Shaker at room temperature at 800 rpm for 30 minutes for primary antibodies and at 750 rpm for secondary antibody and streptavidin.

1. 200 µL flow buffers to each well was added.
2. The plate was incubated on a Heidolph™ Titramax Vibrating Platform Shaker at room temperature at 750 rpm for 30 minutes.
3. Plate was placed on a magnet for 1 minute.
4. The supernatant was emptied in the sink and the plate was tapped three times on paper. The plate was removed from the magnet.

100 µL of sheet fluid was added to each well and the plate was placed on Heidolph™ Titramax Vibrating Platform Shaker at room temperature at 750 rpm for 5 minutes. The plate was then analysed on Bio-Plex 200 (Bio-Rad).

2.5 Bio-Plex 200: Antibody analysis

The Bio-Plex 200 had to be turned on 30 min before analysing samples to warm up. The Bio-Plex program, Bioplex manager, was opened on the computer and start up and calibration programs were performed before samples could be analysed.

2.5.1 Start up and calibrate

A Bio-Plex Calibration kit (Bio-Rad, cat. 171-203060) was taken out from the fridge and put in room temperature for 10 min before calibration. Bio-Plex beads, CAL1 and CAL2 were vortexed for 30 seconds, and 6 drops of each tube were added to an MVC plate. The MVC plate was also added distilled water and 70 % isopropanol before inserted into Bio-Plex 200. In the Bio-Plex program, "Start up & Calibrate" buttons in the quick guide was pushed, and a new window appeared with instructions for calibration (mentioned above). After the calibration process, sheath fluid was added to the fill line and the box with waste liquid was emptied.

2.5.2 Run samples

A new protocol was made where protein coated beads (identified by bead numbers) and plasma samples with control/blank wells were formatted into the Bio-Plex program. The gate valve (DD gate) was set to 5000 – 25 000. Bead region was set to 100 beads minimum for each bead population and sample timeout was set to 60 sec.

When Bio-Plex reading was finished, raw data were exported to Excel and saved. The 96-well plate was placed in a magnet for 60 seconds, the supernatant was emptied in the sink and the plate was tapped three times on a paper. The plate was removed from the magnet and 100 µL of sheet fluid was added to each well. Plate 1-4 (Appendix sec. 6.2) was stored in the fridge and analysed on a different Bio-Plex 200 machine at NVI the day after, resulting in duplets of measurements. Before the plate was analysed, the plate had to be incubated on a Heidolph™ Titramax Vibrating Platform Shaker at room temperature at 750 rpm for 5 min. The machine was calibrated (sec. 2.5.1) and samples were run (sec. 2.5.2). The mean fluorescens intensity (MFI) values used in results originated from the 1st run at the same Bio-Plex 200 machine at NMBU. After analysing samples, wash between plates and shut down procedures were completed, where the machine provided instructions for implementation.

2.5.3 Wash and adjust needle

The Bio-Plex sample collection needle was washed once a month to avoid aggregation and slow count of beads. Distilled water was pressed through the needle with the use of a 50 mL syringe, followed by 10 percent bleach and a second round with distilled water. The needle was sonicated in cold water for 15 min and placed back into the Bio-Plex machine. The needle was adjusted to the wells.

2.6 Statistical analysis

GraphPad Prism 8 software was used for all statistical analysis. Different analyses were run according to whether data were compared within groups (paired) or between groups (unpaired), and according to number of groups compared, and samples (n) in each group.

A parametric paired T-test was run to compare aggregation and bead count between two runs on two different Bio-Plex 200 machines in figure 3.2 where n = 96 in each group. A non-parametric Friedman (paired) test was run to compare the effect on bead count on antibody measurements from three runs in figure 3.3 where n = 8. A non-parametric Wilcoxon (paired) test was run to compare antibody levels detected from fish in the four different experimental groups (PRV-1, PRV-2 PRV-3, InPRV-1) in figure 3.4-3.7 where n=8. When the number of samples per group were n = 8 as in figure 3.3 – 3.7, the test was non-parametric as it could not conclude that data were normally distributed. It was considered that a p-value of ≤ 0.05 were significant and asterisks were used to differentiate between p-values (table 2.5).

Table 2.5. Symbols to show significance and no significance (ns) in accordance with P-value. *Asterisks symbols were used in some figures to differentiate between levels of significance.*

Symbol	*	**	***	****
Meaning	*$P \leq 0.05$*	*$P \leq 0.01$*	*$P \leq 0.001$*	*$P \leq 0.0001$*

3.0 Results

When interpreting the results in this section, significantly higher binding of antibodies to beads coated with PRV antigens (PRV-1 σ1-LM, PRV-1 µ1C, PRV-3 µNS, PRV-3 σ1) than to control beads coated with non-PRV antigens (ISAV FP, ISAV FP-LM) will be referred to as PRV-specific antibodies, based on previous work (116). Binding to PRV antigens have been compared with binding to their most relevant control antigen in the same fish, using paired statistical analyses: Binding to PRV-1 σ1-LM was compared with ISAV FP-LM, both being lipid modified. Binding to PRV-1 µ1C, PRV-3 µNS, PRV-3 σ1 were compared with ISAV FP, not lipid modified. Binding of antibodies to control beads are defined as unspecific antibodies (polyreactive antibodies) (116). These antibody data and additional data from this trial have been published (attachment 6.9) (162). Relevant discussion of the antibody results in relation to virus levels, disease pathology and effects of immunization will be further addressed in the discussion.

3.1 Detection of Anti-PRV antibodies (IgM)

The trial period was divided in two periods based on the introduction of shedders infected by PRV-1 at 10 wpc (fig. 2.1). Period I was defined as the first 10 weeks of the trial (0-10 wpc) before shedders were introduced to groups. Period II was defined as the last 8 weeks of the trial (10-18 wpc) where shedders infected with PRV-1 was introduced to group 2 (fish immunized with PRV-2), group 3 (fish immunized with PRV-3) and Group 4 (fish immunized with InPRV-1). PRV-1 (positive control) was not introduced for PRV-1 shedders.

3.1.1 Detection of PRV specific antibodies (IgM) in plasma after PRV-1 infection

Detection of PRV specific antibodies in plasma of Atlantic salmon infected with PRV-1 (positive control) illustrated in figure 3.1. Detection of PRV specific antibodies binding to PRV proteins (PRV-1 σ1-LM, PRV-1 µ1C, PRV-3 µNS and PRV-3 σ1) were compared to control antigens (ISAV FP and FP-LM) at different weeks post challenge (wpc). The PRV and controls were analysed in same fish and the data was therefore paired.

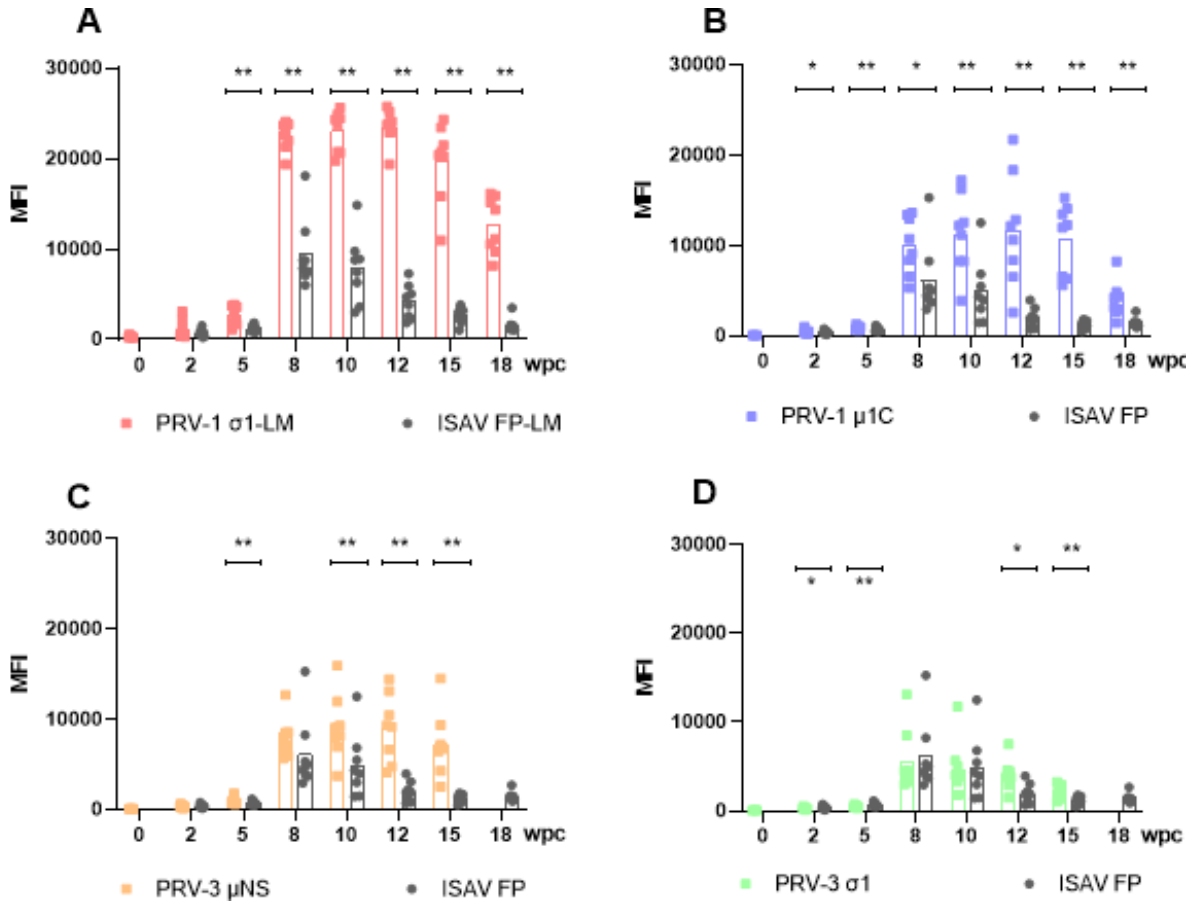

Figure 3.1. Antibody detection in Group 1 (PRV-1). *Detection of antibodies against viral proteins in Atlantic salmon infected with PRV-1 from 0-18 weeks post challenge (wpc). Antibodies against (A) PRV-1 σ1-LM, (B) PRV-1 µ1C, (C) PRV-3 µNS and (D) PRV-3 σ1 shown as mean fluorescence intensity (MFI) for individual fish and bars as group mean. Statistical analysis comparing group mean MFI at each time point the control antigen (grey) being lower (asterisks above) or higher (asterisks below) than the PRV viral proteins using Wilcoxon test, results shown as p-values (* $p \leq 0.05$, ** $p \leq 0.01$). Raw data in Appendix 6.8.1.*

PRV specific antibodies were produced after infection with PRV-1. PRV specific antibodies were detected at 5-18 wpc after PRV-1 infection. Antibodies binding to PRV-1 σ1-LM increased sharply at 8 wpc and remained elevated throughout 12 wpc before gradually decreasing until 18 wpc (fig. 3.1 A). PRV-1 µ1C detected antibodies higher than control beads 2-18 wpc (fig. 3.1 B). In general, antibody levels against PRV-1 µ1C appeared to be lower than observed for PRV-1 σ1-LM. Antibody levels detected on PRV-3 µNS were slightly higher than control levels 5 wpc and 10-15 wpc and could be considered PRV-specific (fig. 3.1 C). Antibody levels binding to PRV-3 σ1 were slightly higher than its control 12-15 wpc and could be considered PRV-specific (fig. 3.1 D).

Unspecific antibodies binding to control beads are still present after heat treatment of plasma. More antibodies bind to the ILAV FP-LM control than to the ILA FP control. Unspecific antibodies binding to control beads are visually detected at its highest at 8 wpc and gradually decreases towards 18 wpc for both controls.

3.1.2 Detection of PRV specific antibodies (IgM) in plasma after PRV-2 immunization

Detection of PRV specific antibodies in plasma of Atlantic salmon immunized with PRV-2 (group 2) illustrated in figure 3.2.

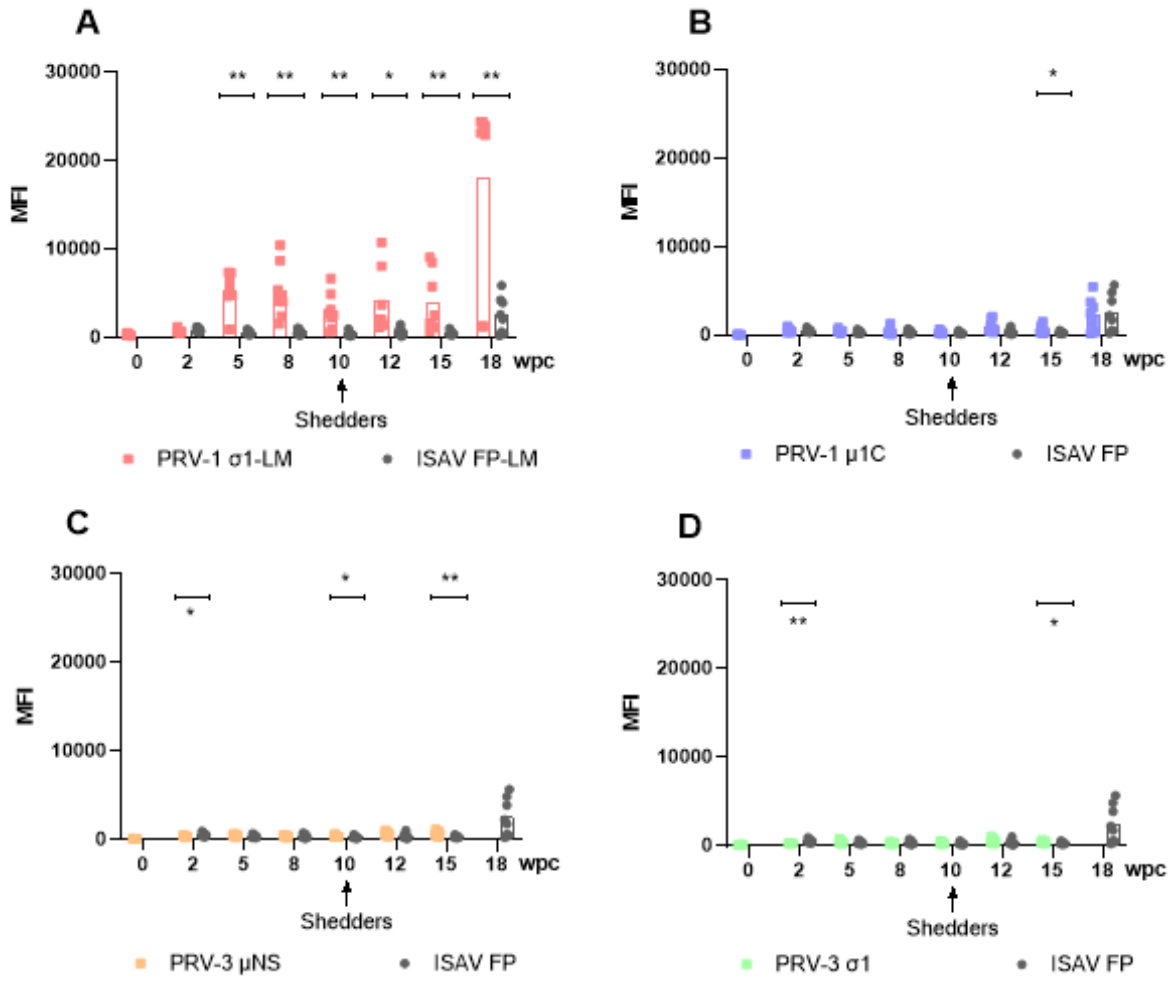

Figure 3.2. Antibody detection in Group 2 (PRV-2). *Detection of antibodies against viral proteins in Atlantic salmon immunized with PRV-2 from 0-18 weeks post challenge (wpc) and shedders introduced 10 wpc. Antibodies against (A) PRV-1 σ1-LM, (B) PRV-1 µ1C, (C) PRV-3 µNS and (D) PRV-3 σ1 shown as mean fluorescence intensity (MFI) for individual fish and bars as group mean. Statistical analysis comparing group mean MFI at each time point the control antigen (grey) being lower (asterisks above) or higher (asterisks below) than the PRV viral proteins using Wilcoxon test, results shown as p-values (* $p \leq 0.05$, ** $p \leq 0.01$). Raw data in Appendix 6.8.2.*

PRV specific antibodies were produced after immunization with PRV-2, and binding to PRV-1 σ1-LM at 5-10 wpc (fig. 3.2 A). In contrast, no PRV specific antibodies were detected to bind to PRV-1 µ1C or PRV-3 σ1 at 2-10 wpc (fig. 3.2 B, D). Furthermore, no PRV specific antibodies were detected to bind to PRV-3 µNS, except a minor increase in specific antibodies binding at 10 wpc (fig. 3.2 C).

Following introduction of PRV-1 shedder at 10 wpc, antibodies against PRV-1 σ1-LM were detected from 12-18 wpc. Interestingly, the antibody levels increased sharply at 18 wpc (fig. 3.2 A). PRV-1 µ1C detected antibody levels higher than control beads 15 wpc after PRV-1 shedder introduction. No PRV specific antibodies were detected to bind to PRV-3 σ1 after addition of PRV-1 shedders. A small significant increase in PRV specific antibodies binding to PRV-3 µNS was detected at 15 wpc (fig. 3.2 C).

Minor amounts of unspecific antibodies were detected on control beads after PRV-2 immunization. Detection of unspecific antibody levels visually increased 18 wpc after PRV-1 shedder introduction.

3.1.3 Detection of PRV specific antibodies (IgM) in plasma after PRV-3 immunization

Detection of PRV specific antibodies in plasma of Atlantic salmon immunized with PRV-3 (group 3) is illustrated in figure 3.3.

PRV specific antibodies are produced after immunization with PRV-3, and binding to PRV-1 σ1-LM at 5-10 wpc. Specific antibodies attached to PRV-1 σ1-LM were detected 5 wpc and increased 8 wpc where it remained elevated throughout 10 wpc (fig. 3.3 A). Even though PRV-1 shedders were introduced 10 wpc, no increase in PRV-1 specific antibody levels were detected. PRV specific antibodies binding PRV-1 σ1-LM gradually decreased from 12-18 wpc.

PRV specific antibodies were binding to PRV-1 µ1C at 2 wpc and 12 wpc (fig. 3.3 B). Significantly higher binding to PRV-3 µNS beads were detected at one time point (fig. 3.3 C). No PRV specific antibodies bound to PRV-3 σ1 (fig. 3.3 D).

Minor amounts of unspecific antibodies, but somewhat higher than PRV-2, were detected on control beads after PRV-3 immunization. Detection of unspecific antibody slightly increased 8-10 wpc, but remained low after PRV-1 shedder introduction.

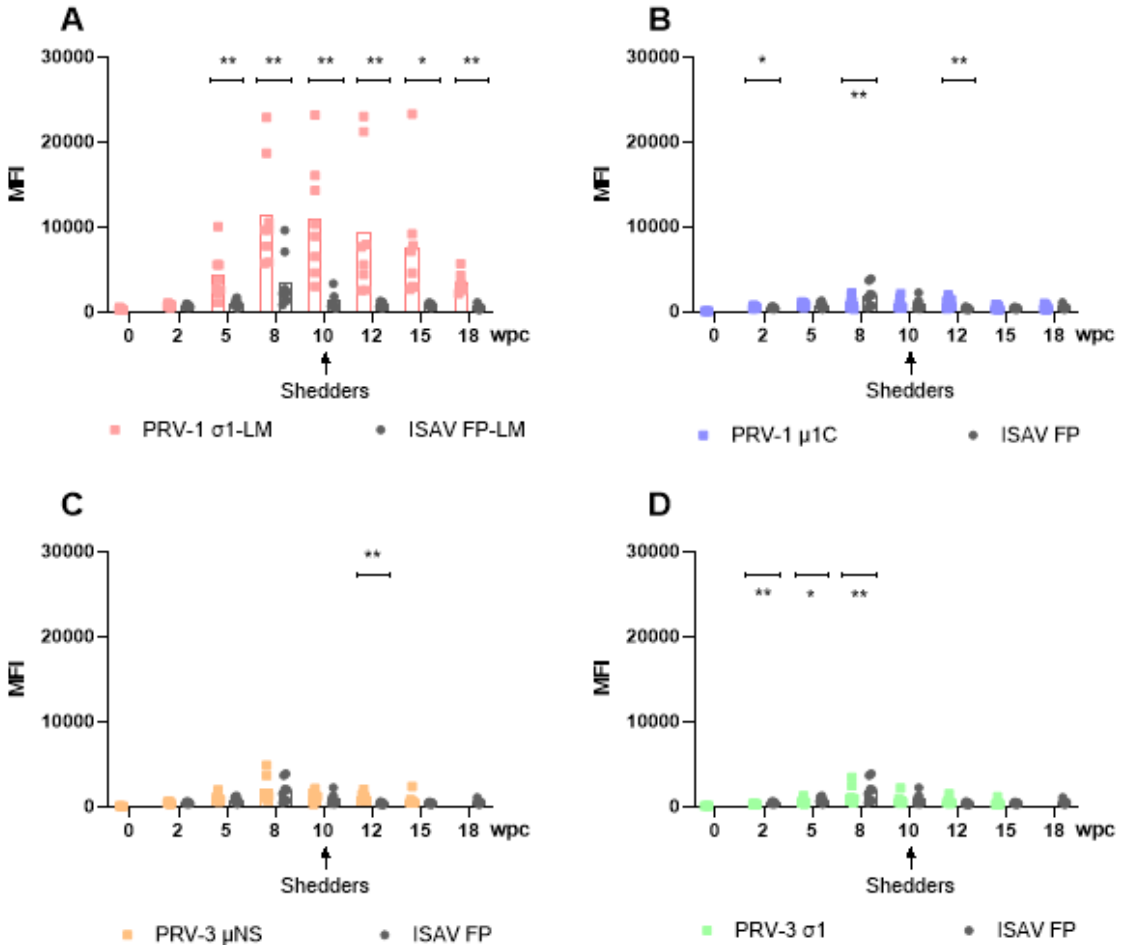

Figure 3.3. Antibody detection in Group 3 (PRV-3). *Detection of antibodies against viral proteins in Atlantic salmon immunized with PRV-3 from 0-18 weeks post challenge (wpc) and shedders introduced 10 wpc. Antibodies against (A) PRV-1 σ1-LM, (B) PRV-1 µ1C, (C) PRV-3 µNS and (D) PRV-3 σ1 shown as mean fluorescence intensity (MFI) for individual fish and bars as group mean. Statistical analysis comparing group mean MFI at each time point the control antigen (grey) being lower (asterisks above) or higher (asterisks below) than the PRV viral proteins using Wilcoxon test, results shown as p-values (* $p \leq 0.05$, ** $p \leq 0.01$). Raw data in Appendix 6.8.3.*

3.1.4 Detection of PRV specific antibodies (IgM) in plasma after InPRV-1 immunization

Detection of PRV specific antibodies in plasma of Atlantic salmon immunized with InPRV-1 (group 4) illustrated in figure 3.4.

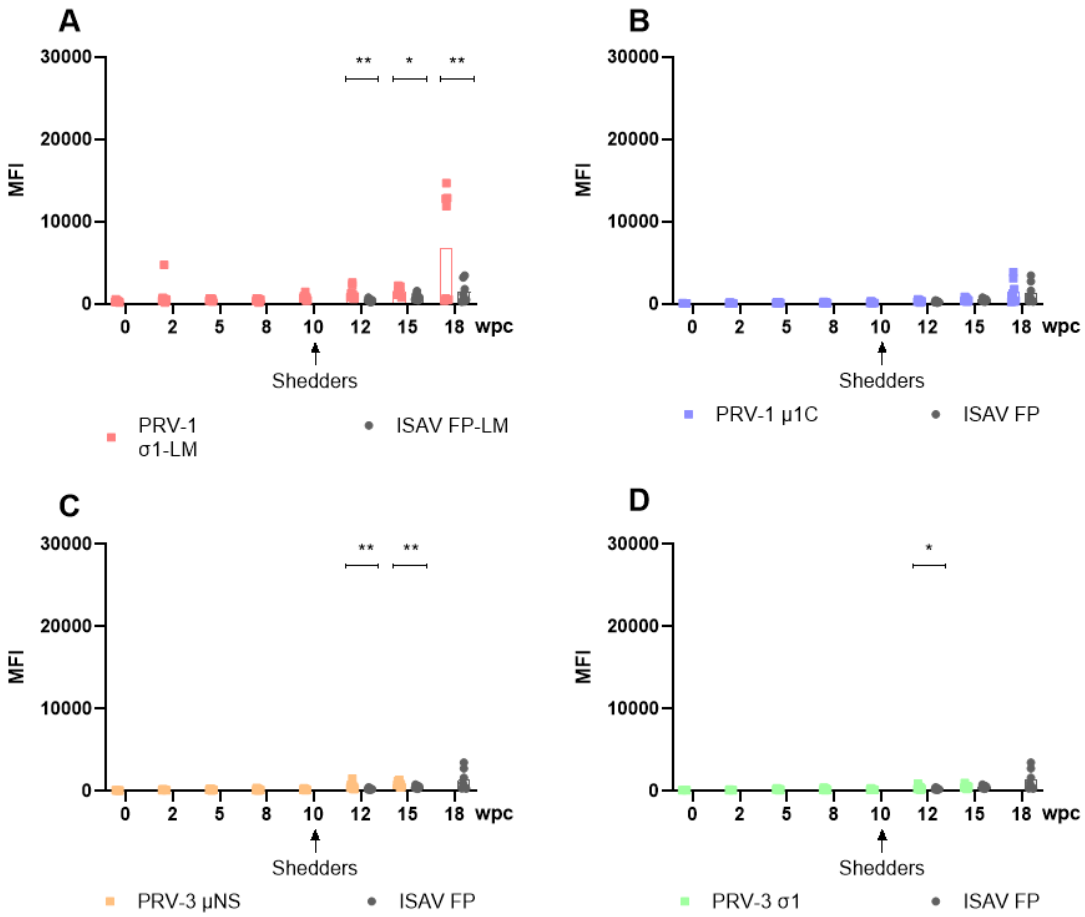

Figure 3.4. Antibody detection in Group 4 (InPRV-1). *Detection of antibodies against viral proteins in Atlantic salmon immunized with InPRV-1 from 0-18 weeks post challenge (wpc) and shedders introduced 10 wpc. Antibodies against (A) PRV-1 σ1-LM, (B) PRV-1 µ1C, (C) PRV-3 µNS and (D) PRV-3 σ1 shown as mean fluorescence intensity (MFI) for individual fish and bars as group mean. Statistical analysis comparing group mean MFI at each time point the control antigen (grey) being lower (asterisks above) or higher (asterisks below) than the PRV viral proteins using Wilcoxon test, results shown as p-values (* $p \leq 0.05$, ** $p \leq 0.01$). Raw data in Appendix 6.8.4.*

PRV specific antibodies could not visually be detected in plasma of Atlantic salmon immunized with inactivated PRV-1 up to 10 wpc. Following the introduction of PRV-1 shedders at 10 wpc, the levels of PRV specific antibodies increased compared to the control antigen at 12-18 wpc (fig. 3.4 A). A similar tendency was also observed against the other PRV antigens tested. PRV specific antibodies were also detected to bind PRV-3 µNS and PRV-3 σ1 afer PRV-1 shedder introduction (fig. 3.4 C, D). No PRV specific antibodies bound to PRV-1 µ1C (fig. 3.4 B).

3.1.5 Detection of PRV-1 specific antibodies (IgM) between groups

Detection of antibodies from plasma of Atlantic salmon binding to PRV-1 σ1-LM was compared between groups divided in period I (fig. 3.5 A) and period II (fig. 3.5 B). Negative control group was divided into two groups at 10 wpc, where one group was introduced to shedders (group 5b) and one group was not introduced to shedders (group 5a) (fig. 3.5 B).

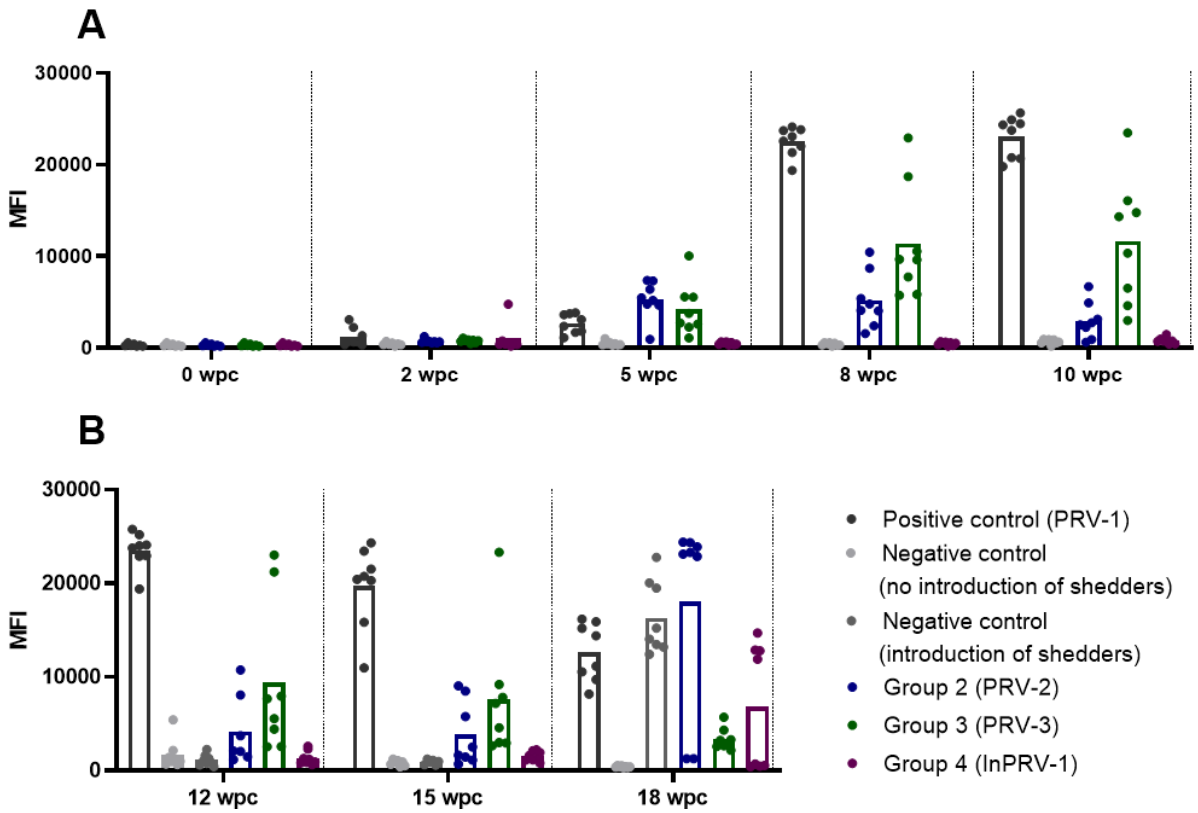

Figure 3.5. Antibody detection between groups. *Detection of antibodies in period I before shedder introduction 10 wpc (A) and period II after shedder introduction 10 wpc (B). Antibody detection shown as mean fluorescence intensity (MFI) for individual fish and bars as group mean. Positive control not added shedders 10 wpc. Raw data in Appendix 6.8.*

In period I, detection of PRV specific antibodies binding to PRV-1 σ1-LM was higher in fish infected with PRV-1 (positive control) compared to all other groups at 8-10 wpc. Fish immunized with PRV-3 had relatively higher detection of PRV specific antibodies binding to PRV-1 σ1-LM than fish immunized with 2 at 5-10 wpc. Antibodies first appeared to bind PRV-1 σ1-LM at 2 wpc from two fish from positive control and one fish from fish immunized with PRV-3. At 5 wpc, PRV specific antibodies binding to PRV-1 σ1-LM from fish immunized with PRV-3 and PRV-2 was slightly higher visually than PRV specific antibodies binding to PRV-

1 σ1-LM in positive control. Fish immunized with InPRV-1 and negative group had no visual detection of PRV specific antibodies binding to PRV-1 σ1-LM at 5-10 wpc. Interestingly, at 2 wpc antibodies binding to PRV-1 σ1-LM were visually detected in one fish from fish immunized with InPRV-1. No PRV specific antibodies appeared to bind to PRV-1 σ1-LM before trial start at 0 wpc (fig. 3.5 A).

For period II, introduction of PRV-1 shedders did not increase (boost) binding of PRV specific antibodies to PRV-1 σ1-LM in fish immunized with PRV-3. PRV specific antibodies binding PRV-1 σ1-LM in fish immunized with PRV-3 was reduced from 12-18 wpc. This was also seen for fish injected with PRV-1 in period I, which were not introduced to shedders. At 12 wpc and 15 wpc, two fish and one fish had higher levels of antibodies binding to PRV-1 σ1-LM than the rest of the samples in fish immunized with PRV-3. At 18 wpc, PRV specific antibodies binding PRV-1 σ1-LM from fish immunized with PRV-3 was relativly lower than the other groups. After week 10, only fish immunized with PRV-2 and InPRV-1 had an increase in PRV-specific antibodies.

3.2 Measurement of PRV specific antibodies on two Bio-Plex 200 machines

3.2.1 Effect of aggregation on bead loss

Plasma samples were run on two different Bio-Plex 200 machines to evaluate if the machines gave the same assay response to be able to transfer the PRV antibody assays to the Veterinary institute (VI). The reanalysis of the plates, 2nd run at VI, gave increased aggregation (fig. 3.6 B) and a lower bead count (fig. 3.6 A).

A higher aggregation and lower bead count from 2nd run resulted in plasma samples only from 1st run at NMBU to be used in analysis in sec. 3.2. This because plasma samples run 1st time on Bio-Plex had a higher bead count and lower aggregation than 2nd run on Bio-plex.

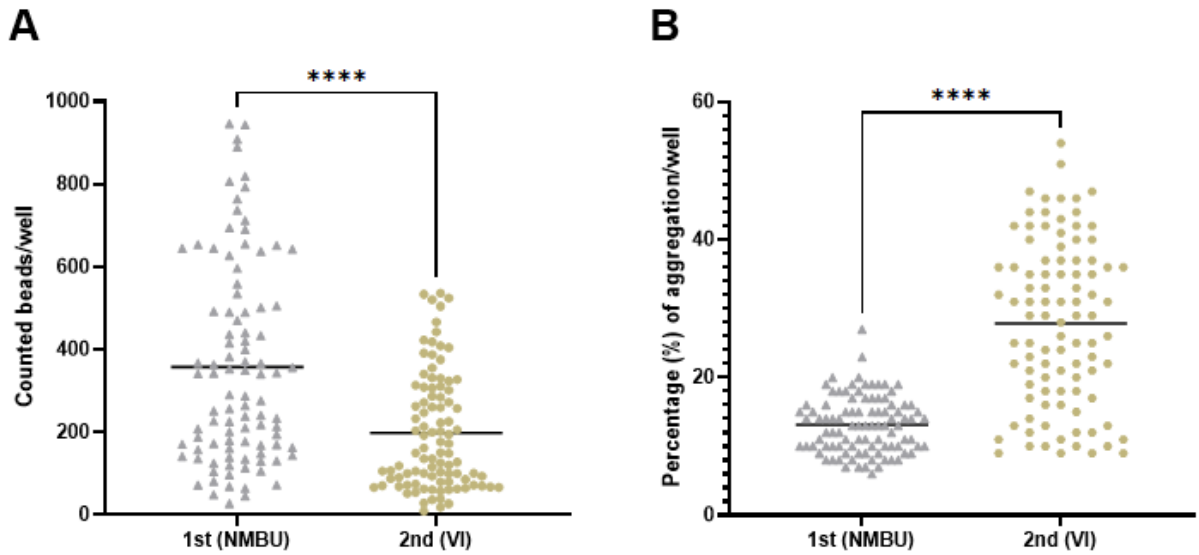

Figure 3.6. Aggregation and bead count after reanalysis on two different Bio-Plex 200 machines. *Measuring the number of counted beads (A) and % aggregation of beads (B) for each plasma sample (well) at the 1st and 2nd run at two different Bio-Plex 200 machines, 1st at NMBU (grey) and 2nd at Veterinary institute (VI) (brown). Statistical analysis comparing the group means of 1st and 2nd run using a paired T-test, results shown as p-values (**** $p \leq 0.0001$). Raw data in Appendix 6.6.*

3.2.2 Effect of bead count on antibody measurement

Plasma samples were run three times (NMBU, VI, VI) on two different Bio-Plex 200 machines to evaluate if a cut off on 10 beads per bead population would influence antibody measurement (MFI). The three runs showed no significant difference on antibody measurement (fig. 3.7 A), but differences in bead count as illustrated in fig. 3.6 A and 3.7 B. No significant difference in antibody measurement with an average bead count of 66, 37 and 8 for each bead population defended a bead count cut off on 10 beads of each bead population per well. Samples (wells) with 10 or more beads counted from each bead population was therefore included in results in sec. 3.1.

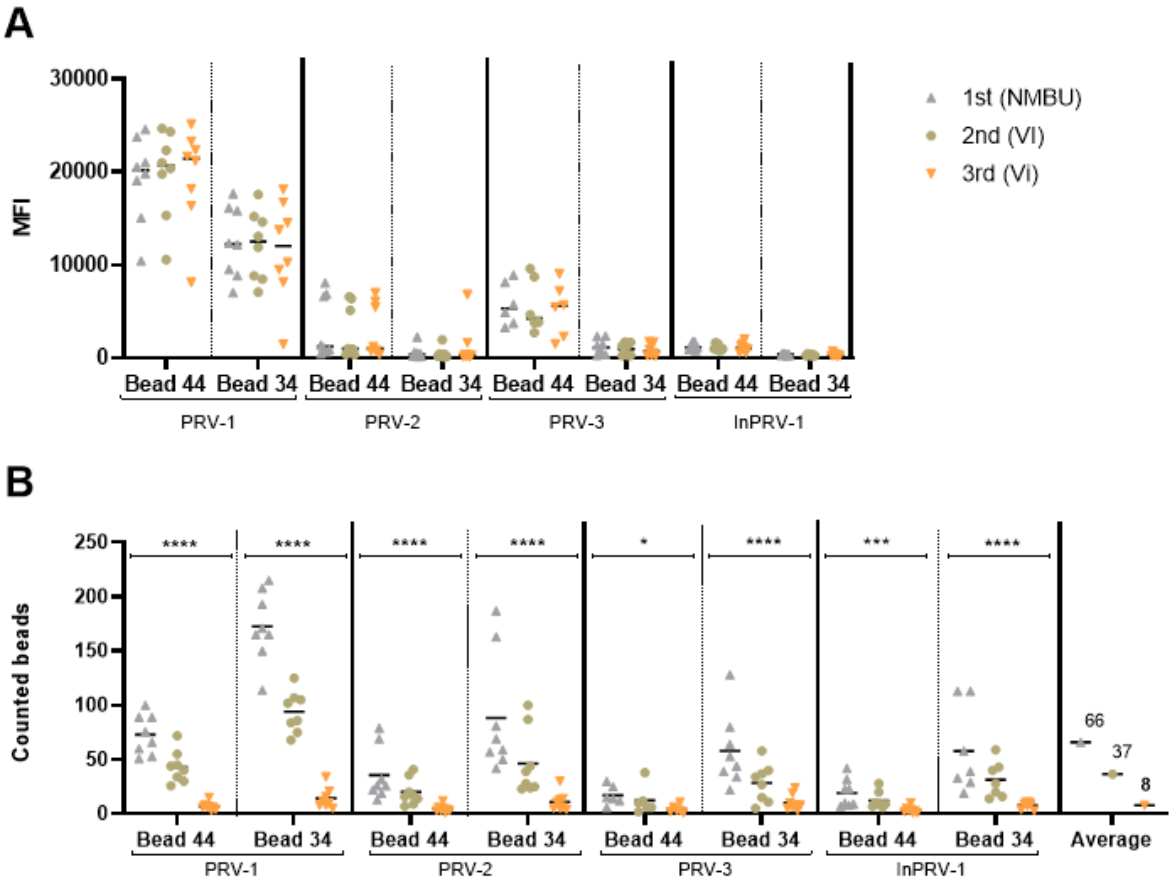

Figure 3.7. Effect of bead count on antibody measurement in the different study groups*. Mean fluorescence intensity (MFI) from PRV-1 bead 44 (PRV-1 σ1-LM) and PRV-1 bead 34 (PRV-1 μ1C) (A) and counted PRV-1 beads 44 and PRV-1 bead 34 (B) during three runs on two different Bio-Plex machines, once at NMBU (blue) and twice at the Veterinary institute (VI) (green, orange). Statistical analysis compares the medians of the three runs using a Friedman test, results shown as p-values (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Raw data in Appendix 6.7.*

3.2.3 Investigating the bead loss during bead coating

Bio-Rad do not offer premade bead kits with antigens against PRV. Therefore, our own bead was coated and bead loss during the coating process was investigated.

Coating of antigen to magnetic beads was done twice, where only the 1st set of coating was used for the Bio-Plex analysis (fig. 3.7 A). The bead count after coating varied between bead populations and a bead loss range from 5 percent – 52 percent was observed. Around half (45 percent - 52 percent) of bead numbers 27 from 1st set of coating and bead 34 from both sets of coating were lost during bead coating. The quality of coating is unknown as there are no methods for quantifying proteins coated to beads.

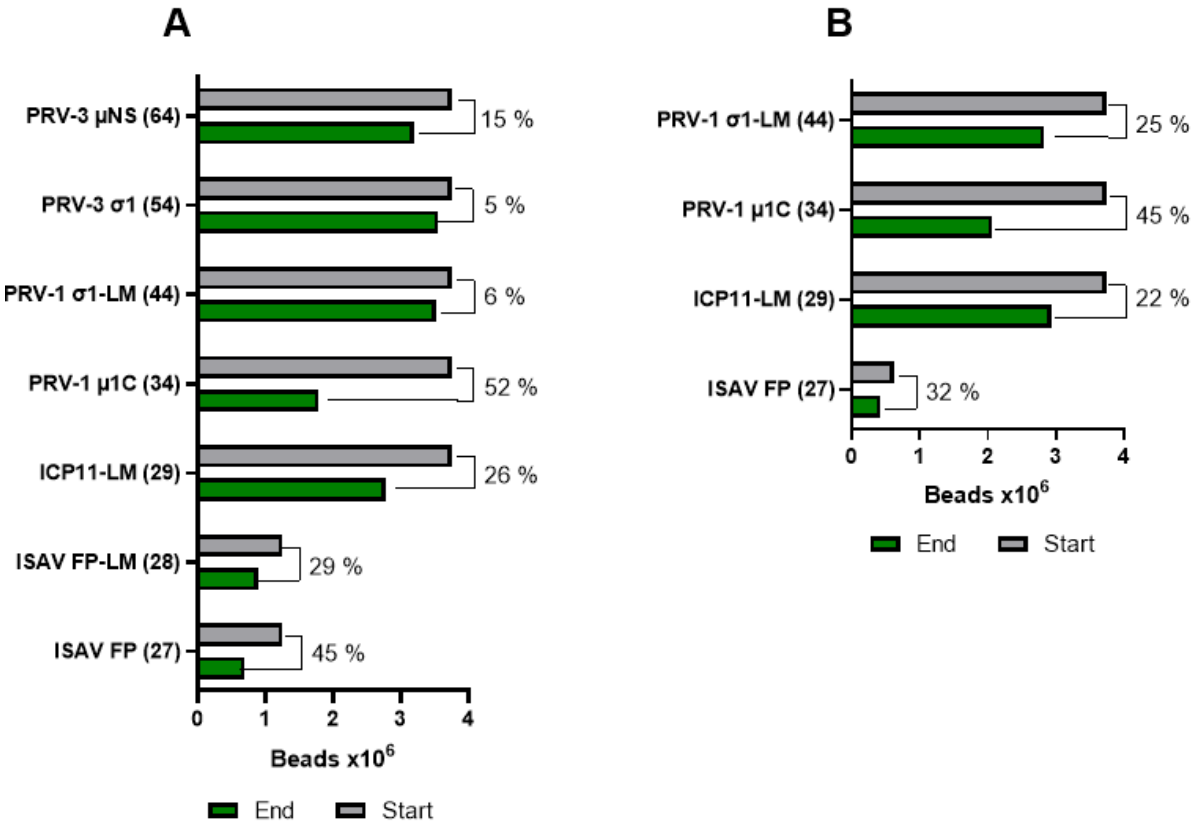

Figure 3.8. Investigating the bead loss during bead coating. *Beads at the start (grey) and at the end (green) of the bead coating with calculated bead loss (%). Different beads added at the start in regard to coupling reaction (x0.5, x1, x3) and available protein. One coupling reaction (x1) equalled 1.25 x 10^6 beads. Bead loss (%) during coating process for (A) 1st coating of beads used in Bio-Plex analysis and (B) 2nd coating of beads not used in Bio-Plex analysis. Calculation of bead loss (%) in Appendix sec. 6.3.2.*

4.0 Discussion

4.1 Detection of antibodies binding to PRV and control antigens

4.1.1 Overview of antibody production in the immunization trial

This trial was divided in two periods, period I and period II. Period I was the first 10 weeks of the trial (0-10 wpc) before shedders were introduced to any groups. This meant that antibodies measured in period I were produced after injection of PRV-1, PRV-2, PRV-3 and inactivated PRV-1. Period II was the last 8 weeks of the trial (10-18 wpc) where PRV-1 shedders were introduced to fish immunized with PRV-2, PRV-3, InPRV-1 and to non-immunized controls. This meant that antibodies measured in period II could derive from immunization by PRV-2/PRV-3 but are most likely a result of infection with PRV-1. The level of antibodies produced at this stage will also be affected by protection from PRV-1.

The PRV-1 infected group (group 1, Figure 3.1) was not introduced to PRV-1 shedders, and therefore showed the full 18-week time course of antibody production in response to PRV-1. This group acted as a positive control for the levels of PRV specific antibodies produced during a PRV-1 infection in Atlantic salmon, which is the natural host for PRV-1. In fish infected with PRV-1, antibodies were produced and detected by binding to the PRV-1 σ1-LM antigen at 5-18 wpc and PRV-1 μ1C antigen already from 2 wpc. This could indicate a more rapid production of antibodies towards PRV-1 μ1C than towards PRV-1 σ1 (fig. 3.1 A, B) (sec. 4.1.3).

The production of PRV specific antibodies measured in plasma using this assay are free-unbound antibodies, and the level seems to be stable 8-15 wpc. This means that the total amount of antibodies produced are not measured, as some antibodies are bound up in complexes or taken up in cells. The stable antibody level measured at 8-15 wpc may be due to a continuous production of PRV specific antibodies. Here, the production of antibodies and binding/uptake of antibodies to antigens/cells seem to be in equilibrium. However, membrane bound antibodies are also possible to identify and quantify by serological antibody analysis even though this wasn't completed in this master thesis (183). Binding/uptake of antibodies related to uptake into phagocytic cells, where antibody are degraded may also play a role (120). The antibody production appeared to dab off at 18 wpc. PRV specific antibodies binding to PRV-3 antigens will be discussed in section 4.1.4.

ISAV FP and ISAV FP-LM were added to the assay as control antigens. They worked as controls, as the fish immune system had never encountered ISAV before. For fish infected with

PRV-1 there was an increase in unspecific antibodies binding to ISAV FP and ISAV FP-LM. This will be more discussed in sec. 4.1.5.

PRV specific antibodies produced in fish immunized with PRV-2 and PRV-3 was found to bind PRV-1 antigens (cross-binding antibodies). This will be more discussed in sec. 4.1.2.

Fish immunized with PRV-2 (group 2) produced PRV specific antibodies binding to PRV-1 σ1 at 5-10 wpc, and at 12-18 after PRV-1 challenge (fig. 3.2 A, B). No PRV specific antibodies binding to PRV-1 μ1C were detected from fish immunized with PRV-2, but PRV specific antibodies were detected at 15 wpc after PRV-1 challenge (fig. 3.2 B). The antibodies detected in period I to bind to PRV-1 σ1 indicated a specific, but low antibody response towards PRV-2. After PRV-1 shedders were introduced, an increase in the production of antibodies detected by PRV-1 σ1-LM were seen (fig. 3.2 A).

Fish immunized with PRV-3 (group 3) produced PRV specific antibodies binding to PRV-1 σ1 at 5-10 wpc, and after PRV-1 challenge at 12-18 wpc (fig. 3.3 A, B). However, PRV specific antibodies binding to PRV-1 μ1C were only detected at 2 wpc after immunization, and after PRV-1 shedder challenge at 12 wpc (fig. 3.3 B). PRV specific antibodies did not bind to PRV-3 μNS or PRV-3 σ1 after immunization with PRV-3 (3.3 C, D). One would expect even more antibodies to be produced and bind PRV-3 antigens (if working in this assay) than to PRV-1 antigens, as PRV-3 was injected in fish. This indicated that the PRV-3 antigens coated on beads were not optimal for binding of PRV-specific antibodies (sec. 4.1.4).

For immunization with inactivated PRV-1 (Group 4, fig. 3.4), PRV specific antibodies were only detected after PRV-1 challenge 10 wpc and not induced by immunization. This will be discussed in sec. 4.2.3.

4.1.2 PRV-1 σ1-LM is suited to detect specific antibodies towards all subtypes of PRV

Detection of specific antibodies towards PRV-1 σ1-LM was seen after PRV-1 (group 1), PRV-2 (group 2) and PRV-3 (group 3) injection (fig. 3.1-3.3). PRV-1 σ1-LM has been confirmed in another trial to be well suited for detection of specific antibodies against PRV-1 in plasma (116). This was confirmed when PRV-1 σ1-LM showed similar binding of antibodies in heat-treated plasma. For the control proteins used (ICP11-LM, ISAV FP, ISAV FP-LM), there was a lower antibody binding after heat-treatment of plasma compared to the non-heat-treated plasma. This indicated that the heat treatment decreased binding of unspecific/polyreactive antibodies on control beads. Therefore, PRV-1 σ1-LM was used as the main antigen for

detecting specific antibodies in this trial, and compared within (fig. 3.1-3.4) and between groups (fig 3.5) (116).

In this thesis, antibodies induced by different PRV subtypes were shown to cross-bind PRV-1 σ1-LM. Cross-binding antibodies rely on similarities in amino acid sequence and/or 3D structure and surface charges. PRV-3 have an amino acid resemblance to PRV-1 of 90.5 percent, while PRV-1 and PRV-2 have an amino acid resemblance of 80.3 percent (164). Antibodies are produced towards antigen based on both amino acid sequence (similar proteins) and structure of antigen. When similar antigens are presented to fish, antibodies produced may cross-bind as they do for PRV subtypes. The outer capsid protein PRV-1 σ1 has an amino acid resemblance of 81.6 percent and 66.7 percent towards PRV-3 σ1 and PRV-2 σ1, respectively (164).

Despite that the amino acid resemblance of σ1 between PRV subtypes is lower than the overall similarity, PRV subtypes could cross-bind σ1. Probably because of the similarity in structure of σ1 when coated on beads (likely due to the lipid modification). The antibodies detected on PRV-1 σ1-LM could therefore be compared between groups in section 4.2 and 4.3 (fig. 3.5). However, PRV specific antibodies from fish immunized with PRV-2 and PRV-3 might have a lower ability to cross-bind antigen than PRV specific antibodies from fish infected with PRV-1.

4.1.3 Why did PRV-1 μ1C not pick up cross-binding antibodies?

In this section, different points on why PRV-1 μ1C didn't detect cross-binding antibodies from fish immunized with PRV-2 and PRV-3 will be discussed.

PRV specific antibodies towards PRV-1 μ1C were detected at 2-18 wpc from fish infected with PRV-1 and at 2 wpc from fish immunized with PRV-3. In addition, PRV specific antibodies towards PRV-1 μ1C were detected after PRV-1 shedder introduction. Specific antibodies binding PRV-1 μ1C have been detected by Western blot in plasma from PRV-1 infected Atlantic salmon in another study (132). These specific antibodies were not detected in uninfected control fish. This is an additional proof that PRV specific antibodies, as detected in this assay, are present in plasma (132). Antibodies targeting PRV-1 μ1C and PRV-1 σ1 has also showed to recognize PRV-3 μ1C and PRV-3 σ1 during western blotting. Showing that antibodies produced towards PRV-1 μ1C and PRV-1 σ1 could cross-bind PRV-3 μ1C and PRV-3 σ1 (160). However, PRV-1 μ1C coated to beads did not show the same possibility in cross-binding throughout the trial for fish immunized with PRV-2 and PRV-3. There might be

many reasons for this, but knowing that PRV specific antibodies are produced (132) and can cross-bind PRV-3 µ1C (160) it indicates that the reason may be correlated to how PRV-1 µ1C is coated to beads (structure). Antigens coated to beads function best if they mimic antigens as they are presented in fish. This to make antibodies recognize the protein and to make antibody binding correlate to the number of free-unbound antibodies actually produced towards PRV-1 µ1C in fish. Differences in detection of antibodies between groups immunized with different PRV subtypes might indicate several points.

Firstly, the folding of PRV-1 µ1C in Atlantic salmon might be different than the folding of PRV-1 µ1C in rainbow trout, compared to the folding of PRV-1 µ1C produced in *E.coli*. This because antibodies produced towards PRV-1 µ1C was detected when produced in rainbow trout (160), but not in this experiment when produced in Atlantic salmon.

Secondly, the quantity of protein coated to each bead could also affect levels of antibodies detected. Less PRV-1 µ1C coated to beads than PRV-1 σ1-LM will give less antibody detection. This will be more discussed in sec. 4.4.2.

Thirdly, the PRV-1 outer capsid protein µ1 has an amino acid resemblance of 91.5 percent and 85.1 percent to PRV-3 µ1 and PRV-2 µ1, respectively (164). Interestingly, this would in theory mean that PRV-1 µ1 should bind more cross-binding antibodies (if produced) than PRV-1 σ1. However, the structure of the antigens is of importance for antibodies to recognize antigens. The importance of lipid modification of PRV-1 σ1 and how it sits on bead is therefore again reflected (116).

One can discuss if antibody production towards PRV-1 µ1C is actually lower than the antibody production towards PRV-1 σ1-LM in PRV-1 injected fish and none detected for PRV-2/PRV-3 injected fish. However, it might also be because of the assay. Comparison between levels of different antibodies (MFI) detecting different antigens may therefore be impossible if detection is related to the assay, and not the actual level. The effect of heat treatment of plasma on antibody levels detected by PRV-1 µ1C could have been performed as Teige did with PRV-1 σ1 in her study (116). If PRV-1 µ1C also were lipid modified, one could be able to evaluate how PRV-1 µ1C is presented on beads with and without modifications. This because one is uncertain about the level of binding compared to the actual level in plasma.

To conclude, there might actually be less antibodies produced towards PRV-1 µ1C as detected in this assay. There might also be produced more antibodies towards PRV-1 µ1C but it is not

detected due to assay or the ability to cross-bind to PRV-1 µ1C (folding of protein in Atlantic salmon versus *E. coli*). A lower affinity is most likely triggered by the structure of the protein when it sits on beads, as amino acid resemblance is relatively high (164). It might also be that the active seat of antibodies target µ1, and when µ1 is cleaved to µ1C and µ1n (144), it decreases the ability of antibody to bind.

It is hard to trust the significant findings at 2 wpc for fish immunized with PRV-3 because there at 8 wpc are shown that control beads bind significantly more antibodies than PRV-1 µ1C (fig. 3.3 B). Because one is uncertain about the level of binding compared to the actual level in plasma in Atlantic salmon infected with PRV-2 and PRV-3, nothing can be concluded regarding PRV specific antibodies in plasma for these groups. For PRV-1 infected group PRV specific antibodies are binding to PRV-1 µ1C. However, one is uncertain how much is specific antibodies, how much is unspecific antibodies and if it is correlated to amount produced in fish.

4.1.4 Why did PRV-3 antigens not pick up specific antibodies?

PRV-3 proteins did not succeed at detecting PRV specific antibodies after immunization with PRV-3. Antibodies produced here should have bound to PRV-3 antigens and not only to PRV-1 antigens. PRV-3 µNS and PRV-3 σ1 targeted antibodies is with high probability produced in plasma, as antibodies has been detected towards PRV-1 µNS and PRV-1 σ1 in Atlantic salmon previously (132, 160). The PRV-3 proteins used here are produced in insect cells and coated to beads. The folding of the proteins in insect cells might be different than the folding of protein in Atlantic salmon. Antibodies produced towards PRV-3 antigens in fish might therefore have problems binding to the PRV-3 proteins on beads, and this may hamper antibody binding from fish immunized also with PRV-2 and PRV-1. However, one expects the protein to be folded correctly. Firstly because PRV-1 µNS detecting antibodies in Teiges study was produced in insects cells (132). Secondly, because proteins are folded in a eukaryote cell and with a lower temperature than for production in *E.coli* (closer to the temperature of fish) (184).

Another trial completed by Teige (116) revealed that PRV-1 σ1 could not bind PRV specific antibodies in plasma from fish infected with PRV-1. However, when PRV-1 σ1 was lipid modified (PRV-1 σ1-LM) it could bind PRV specific antibodies. This therefore indicated that the reason for why PRV-3 σ1 and probably PRV-3 µNS did not bind antibodies was because of how they were coated to beads.

So, can significant differences observed in antibody binding between control beads and PRV-3 antigen coated beads be trusted? At some time points PRV-3 coated beads bind low but

significantly more antibodies than control beads, and at other time points control beads bind significantly more antibodies. This makes it hard to trust the significant findings using the PRV-3 antigens.

4.1.5 Unspecific antibodies binding ISAV FP and ISAV FP-LM

There was visually a higher detection of polyreactive antibodies binding to ISAV FP and ISAV FP-LM in PRV-1 infected fish compared to the other groups (fig. 3.1). Fish had never encountered ISAV and therefore only unspecific antibodies were detected on the ISAV antigens. Teleost have unspecific natural antibodies that are presented without having encountered an antigen (81). Their role is unclear, where they have been found in serum of fish earlier (113), and stated to often measured as background noise in assays (101), as also been seen in this trial. Teleost have also unspecific antibodies (polyreactive antibodies) that can be secreted after encounter with pathogen. This is seen after PRV-1 infection in Teiges study (116) and also in this study after PRV-1 infection. The ratio between natural antibodies binding and how much polyreactive antibodies binding may be evaluated by looking at mock group. However, since plasma is heat treated as unspecific binding was something we did not want to measure, it is hard to discuss as one is unsure how much unspecific antibodies are removed during heat treatment.

Teleost rely on the innate immune system for an extended period until the adaptive immunity is kicked off (80, 81). Atlantic salmon might produce polyreactive antibodies as affinity maturation process to produce PRV specific antibodies takes time (118). Also seen in this trial slightly detected at 5 wpc (fig. 3.1 A). To cope with PRV-1 in the meantime polyreactive antibodies are produced. Producing a lot of antibodies has its advantage. This is because antibodies can agglutinate viruses and label them for more effectively phagocytosis and removal (120). They can also opsonize pathogens for complement activation which can lyse and kill the virus, but this is more relevant for membrane-coated viruses (130, 133). In addition, NK-like cells can be activated by IgM through antibody-dependent cell-mediated cytotoxicity (ADCC), and degranulate and kill infected host cells (129). Antibodies can also neutralize and block the virus from entering the host cell and do harm (47, 120). These advantages are however only relevant if polyreactive antibodies bind PRV antigens. Polyreactive antibodies can bind and react to a number of unrelated antigens, and even cause autoimmunity (100, 114, 115, 178).

Interestingly, polyreactive antibodies are first seen to increase at the same time as specific antibodies detected at 8 wpc after PRV-1 infection (fig. 3.1 A). To compare, bacteria have many PAMPs that are recognized by polyreactive antibodies (185), while the most important PAMPs on viruses recognized by the immune system is its genome, which is dsRNA in PRV (34, 47). These polyreactive antibodies binding to ISAV-FP appear to increase because of PRV-1 infection. In addition, polyreactive antibodies from PRV-1 seem to follow the same time course as specific antibody produced increasing at 8 wpc.

There were also minor levels of unspecific antibodies detected after PRV-2 and PRV-3 immunization. This unspecific binding might be produced after immunization by PRV-2 and PRV-3 or it might represent natural antibodies that were there before infection (101). Antibody binding may also be induced by complement or other virus-binding molecules (i.e pentraxins and collectins) that are not removed after heat treatment (47). This type of binding has been checked for in negative group (group 5), which contains the same batch of fish expected to have the same basal immune response (sec. 4.4.4).

4.2 PRV specific antibodies compared between groups – After immunization

In this section, PRV specific antibodies binding to PRV-1 σ1-LM are compared between groups after immunization (fig. 3.5 A). This because antibodies produced towards PRV-2 and PRV-3 are found to cross-bind PRV-1 antigen, and because this is a reliable detection method for PRV-1 specific antibodies (sec. 4.1.2) (116). However, it is not certain that the cross-binding of antibodies is as strong as the PRV specific antibodies from PRV-1 infection. Even though, it is well worth a comparison.

More PRV specific antibodies binding to PRV-1 σ1-LM was detected from fish infected with PRV-1 compared to other groups in period I (fig. 3.5 A). This was probably because an injection of PRV-1 into its main host will cause a stronger infection/replication of virus. Strong infection by PRV-1 is shown to trigger a powerful antiviral response. A strong infection by PRV may therefore be a trigger for strong immunity (168) and may be correlated to higher levels of PRV specific antibodies compared to other groups.

4.2.1 Comparing PRV specific antibodies from fish immunized with PRV-3 and PRV-2

PRV specific antibodies (cross binding antibodies) were detected 5-10 wpc after immunization with PRV-3 and PRV-2 (fig. 3.2 A, 3.3 A, 3.5 A). However, PRV specific antibodies binding to PRV-1 σ1-LM were visually higher for fish immunized with PRV-3 than PRV-2, as shown in figure 3.5 A. The visually higher PRV specific antibodies detected on PRV-1 σ1-LM in PRV-3 immunized fish can have several causes that will be discussed in this section.

Firstly, the higher antibody response from PRV-3 might correlate with higher replication and virus levels in fish. Both PRV-3 and PRV-2 replicated in Atlantic salmon. However, higher virus levels were detected in fish immunized with PRV-3 compared to fish immunized with PRV-2 (Attachment 6.9 in Appendix, fig. 2) (162). The article contains data on virus levels, histopathology, cellular immune responses (CD8α, Granzyme A, IFNγ) and innate antiviral immune responses (Mx, ISG15, Viperin), and includes data from this master thesis on PRV specific antibodies (162). A higher replication rate results in more virus produced and is expected to increase the antibody responses towards virus (186).

Secondly, the injection dose was the same for all fish in the same group, but most likely not comparable between groups, as the virus infectivity could not be measured. PRV RNA measured by qPCR does not directly reflect the infectious dose. The infectivity of the virus preparation is correlated to how the material is treated with regards to temperature, storage,

sonication and what type of tissue (blood, organs), and at which time point of the infection organ/blood were sampled. Sonication is performed to lyse host cells to be able to isolate virus. Proteins from virus may also be affected and destroyed during sonication. This is probably in such small quantities that it is insignificant in the context of the experiment. It is impossible to know how much infectious PRV-1, PRV-2 and PRV-3 are present in each of the doses. This is because qPCR only detects amount of genomes correlating to virus, but does not separate between infective and inactivated virus. More knowledge of virus properties are also required to be able to say more about the infectious dose between groups. If having a susceptible cell line that can be used to measure infectivity in lab, one could say more about infectious dose. The infectious dose within the same group are considered to be identical when injected (homogenized solution). However, biological differences between individuals in the same group may affect the capability of the infectious dose of viruses to infect host cells.

Thirdly, antibodies produced will differ in specificity against PRV-1 σ1 when produced towards PRV-3 σ1 and PRV-2 σ1. Antibodies produced towards PRV-3 are likely to be more similar to antibodies recognizing PRV-1 due to amino acid composition (164). On the other hand, antibodies produced towards PRV-2 are likely to be less similar to antibodies recognizing PRV-1 due to amino acid composition (164) and therefore less antibodies might bind to PRV-1 σ1-LM. A higher level of PRV specific antibody detected in PRV-3 seems realistic.

PRV-3 is more closely related to PRV-1 than PRV-2, and more host cells may therefore be more susceptible to infection by PRV-3, resulting in more virus to replicate in host cells (Attachment 6.9 in Appendix, fig. 2) (162). Therefore showing an increased production of PRV specific antibodies (186).

4.2.2 No PRV specific antibodies detected in fish immunized with InPRV-1

PRV specific antibodies were not visually detected from fish immunized with InPRV-1. No statistical test were run between control beads and PRV antigen coated beads in period I (0-10 wpc), as the controls ISAV FP and ISAV FP-LM were not run for InPRV-1 (fig. 3.4). Therefore, visual observations comparing antibody levels binding to PRV-1 σ1-LM in the InPRV-1 group and the negative control group was done in figure 3.5. However, at 2 wpc, a putative increase in PRV specific antibodies was detected in one fish only. This might be related to an individual difference in response to the immunization or just an accidental error in measurements.

Inactivated virus will not replicate in host cells, and therefore no virus levels were detected to replicate and therefore increase in fish from this group (162). In another trial studying immunization by inPRV-1, virus levels were detected and seen to decrease after injection as expected for inactivated viruses (60). No replication of virus is likely to give a weaker immune response (48), and less formation of antibodies (186). However, adjuvants were added to InPRV-1 to trigger both sides of the immune system (49-51). Adjuvants added in this trial might therefore not be as efficient in activating the adaptive humoral part of the immune system as no PRV specific antibodies were detected after immunization. Inactivation of PRV-1 can also have damaged outer surface proteins and therefore result in antibodies not binding to PRV-1 σ1-LM and PRV-1 μ1C. The exact components of this adjuvant is a company secret of Pharmaq/Zoetis.

4.3 PRV specific antibodies produced after PRV-1 shedder introduction in period II of the trial

In this section, PRV specific antibodies binding to PRV-1 σ1-LM are compared between groups after PRV-1 challenge in period II (12-18 wpc).

4.3.1 Comparing protection from fish immunized with PRV-3 and PRV-2

Even though PRV specific antibodies were detected after immunization by PRV-2 and PRV-3, the responses after PRV-1 challenge 10 wpc differed. These antibodies could be derived from a PRV-1 infection or be absent because the fish were protected from PRV-1.

The PRV specific antibody response from fish immunized with PRV-3 did not seem to be affected by the PRV-1 shedders introduced at 10 wpc (fig. 3.3). On the contrary, the PRV antibody response detected by PRV-1 σ1-LM appeared to decrease in period II (fig. 3.5 B, 3.3 A). PRV-1 infection was not detected in most of the fish 12-18 wpc after PRV-1 shedder introduction (Attachment 6.9 in Appendix, fig. 5). This indicated that immunization with PRV-3 protected against PRV-1 by completely blocking infection, and this was confirmed with histopathology and qPCR (162). This protection could partly be due to the PRV specific antibody response but could also be related to other immune protective mechanisms involving cellular immunity, or innate immunity. At 12 wpc and 15 wpc, two and one fish respectively had a higher PRV specific antibody response compared to the rest of the fish group (fig. 3.3). As already explained, this might be caused by biological differences between individual fish, the susceptibility and replication to virus (186).

The PRV specific antibody response from fish immunized with PRV-2 was induced in period II (fig. 2.1) by the PRV-1 shedders introduced at 10 wpc (fig. 3.2, 3.5 B). Most fish immunized with PRV-2 showed an infection by PRV-1 at 15-18 wpc (Attachment 6.9 in Appendix, fig. 5). The increased production of antibodies was due to an infection of PRV-1, which was confirmed by histopathology and qPCR (162). The PRV specific antibody response visually ranged beyond the levels in the positive control (non-immunized fish infected with PRV-1 shedders in period II) (fig. 3.5 B), and the same did the amount of PRV-1 detected by qPCR compared to positive control (Attachment 6.9 in Appendix, fig. 5). Fish had however a delayed (high Ct value) and variable PRV-1 infection at 12 wpc after PRV-1 shedder introduction (Ct 10-24) in spleen (162), which is expected to reflect the virus levels in blood (136). The variable infection level was also reflected in levels of PRV specific antibodies produced. Two fish did not

respond with an increase in production of PRV specific antibodies, and had a lower virus levels of PRV-1 (162). Five fish had a production of PRV specific antibodies higher than the positive control in period II (fig. 3.5 B). PRV-2 reduced the severity of HSMI in two individuals, but did not block PRV-1 infection (162). The PRV specific antibody response seen in period I was therefore not sufficient to cross-protect against PRV-1. There may however been produced antibodies towards other PRV antigens which are not analysed for in this assay.

4.3.2 Protection of fish immunized with InPRV-1

No PRV specific antibodies were detected to visually bind PRV-1 σ1 from fish immunized with inactivated PRV-1 (fig. 3.1). After introduction of PRV-1 shedders at 10 wpc, PRV specific antibodies were detected 12-18 wpc. Virus levels increased around the same time (15-18 wpc) for all fish after introduction of PRV-1 shedders (Attachment 6.9 in Appendix, fig. 5) (162), but only six fish increased their production of PRV specific antibodies (fig. 3.4, 3.5 B). A partial protection of fish was seen in fish immunized with InPRV-1, where some fish had a lower viral load (Ct-value 30-35) (Attachment 6.9 in Appendix, fig. 5), and six out of eight fish were without heart lesions (162). This was in line with an earlier study performed on this InPRV-1 vaccine candidate, reporting reduced heart lesions but no prevention of PRV-1 infection (60). Since there was no detection of PRV specific antibodies after InPRV-1 immunization in our assay, another mechanism may have protected the heart and prevented HMSI in most fish (162). There may also have been produced antibodies towards other PRV antigens which are not analysed for in this assay.

It is possible that InPRV-1 triggered a local immune response (humoral or cellular immunity) in the peritoneal cavity where the vaccine was injected. Analysis done before PRV-1 shedders were added found no indications of cellular and innate responses in spleen (162). Therefore, little reason to believe that a local non-systemic peritoneal response can protect the heart. The question is then, can B-cells and antibodies produced in peritoneum protect against an infection of PRV-1 and HSMI? Housing of B-cells in the peritoneal cavity might function as a local immune response in response to the InPRV-1 vaccine, as previously seen for a SAV infection (96), without triggering a systemic immune response. This local immune response may not be sufficient to protect from PRV infection and HSMI, since PRV infection is systemic (in red blood cells) (152). However, migration routes are unknown for the B-cells in the peritoneal cavity. It is unknown if the B-cells in the peritoneal cavity can migrate to internal organs or

just induce local immune responses (187). If they migrated, they should (if targeting these assay proteins PRV-1 σ1 and PRV-1 μ1C) have been detected as they would use the blood as a transportation medium to get to organs.

4.4 Measurement of PRV specific antibodies on two Bio-Plex 200 machines

The plasma samples were reanalysed on different Bio-Plex 200 machines, one at NMBU and one at the Veterinary Institute (VI). This was to evaluate if the machines gave the same assay response so PRV antibody assays could be transferred to the VI.

4.4.1 Effect of aggregation on bead loss

There was a lower bead count for the 2nd run of the plasma samples due to an increased occurrence of aggregation in each well (fig. 3.6). Aggregation from PRV-1 (group 1) and Mock (group 5a) from 2-15 wpc (Appendix 6.6) was merged to one figure as no difference in aggregation was seen between groups (not shown in results). A paired T-test was run to compare the mean of aggregation and bead count between the 1st and 2nd run. As the 2nd run gave high aggregation and problems related to low bead count only the 1st run of plasma samples was used as assay results.

According to the Bio-Plex user guide, aggregation is defined as clumping of two or more beads. If beads overlap during Bio-Plex reading, this is calculated as percentage of aggregation of the already (total) counted beads. The light scattered from particles that flow past the red laser is directly proportional to particle size and therefore the machine identifies particles that are smaller (dust, air bubbles) or larger than a single bead (aggregation). If there are few microscopic air bubbles, they can also be read as aggregation. However, air bubbles will cause a sudden shift in the bead region and the reading will stop. This did not occur during assay run and therefore the aggregation detected in wells are most likely actual aggregated beads. In addition, use of lid avoided dust particles to enter the wells.

There was same amount of aggregation of infected PRV-1 plasma and uninfected plasma (not shown in results), and therefore it is not only antibodies that bind antigens that crosslink. Beads aggregate because they in general bind impurities in plasma (lipids, proteins, polysaccharides etc.) that are sticky. They might also get sticky after coating as proteins that are coated are not 100 percent pure.

Less beads were available in each well after 1st count as beads added to each well were limited to 2500 beads of each bead number. However, this should be more than enough to count the minimum limit of 100 beads of each population which Bio-Plex 200 was set to. From 1st and 2nd run there were counted under 100 beads for several wells (fig. 3.6 B). Error 1, 2 and 4 was triggered during this analysis. Error 1 was triggered if there was fewer than 25 percent of the acquired beads per population. This meant that with an acquired bead count of 100 beads per population a low bead count would be triggered if less than 25 beads per population was read. Error 4 was triggered if less than 20 beads were counted from each well (table 2.1). Error 4 indicated that the bead population selected (region selected) was incorrect or that there was actually too few beads in the wells (180). Error 2 was triggered if there was above 50 percent of aggregation in each well. There were no Error 2 triggered during the 1st run and most wells showed under 20 percent aggregation (fig. 3.6 B). For 2nd run there was an increase in aggregation. However, only two samples (wells) contained above 50 percent aggregation and triggered an Error 2 (fig. 3.6 B). Error 3 and Error 5 was not detected during the plate runs.

A lower bead count than the limit of 100 beads set on the computer could correlate to pipetting skills at lab during preparation of the 96-well plate. This because aggregation for 1st run was below 20 percent for most wells and could therefore not be the only reason for a bead count lower than 100 for each bead population. In addition, 60 second is set as sample time. At this point, Bio-Plex 200 will not read more beads and go to next well. For the preparation of the 96-well plate there was a magnet during the washing steps before adding plasma, primary antibody, secondary antibody, and streptavidin and the supernatant was emptied in sink by force. If magnet was too weak to hold on to beads it could be possible that some beads were lost in the sink during these washing steps, and vice versa if the magnetic beads were not strong enough to stay attached to the magnet during the emptying of supernatant.

4.4.2 Sensitivity of detection in relation to bead counts and repeated runs

The analyses were run three times on two different Bio-Plex 200 machines, once at NMBU and then twice at VI to compare the sensitivity of binding analyses at low bead counts. This was to evaluate if a cut off at 10 beads would influence antibody measurement (MFI levels). Bio-Rad recommended a minimum of 50 beads counted per bead population, and a lower bead count error would be triggered if below 20 beads per bead population was counted (180). The

reason for wanting to test a cut off on 10 beads was linked to problems explained in sec. 4.4.1 related to error 2 and 4 (low bead count).
There was no difference measured on MFI for the three runs even though they all showed a decrease in average bead count from 66, to 37 and to 8 beads counted per population (fig. 3.7). Therefore, a bead count cut off on 10 beads per population was possible.

According to the Bio-Plex user guide, each well may be read twice without adversely affecting the results (180). This means that two runs should show a similar MFI value. In this trial, all three runs show similar MFI values. The stability in MFI between runs with decreasing bead count are likely correlate firstly to beads being coated with same amount of protein. Secondly, that plasma added outnumbers all the antigens coated to beads.

Shaking of the plate at HulaMixer is important to homogenize the sample and decrease aggregation. Homogenizing sample increases the possibility that all attachment point of protein binds specific antibodies in plasma if available and produced (taking in account that proteins are coated in a correct manner mimicking the way it is presented in fish).

The blocking buffer added at the end of the coating process make sure that carboxyl groups on beads do not bind unspecific antibodies. The side groups of antibodies were not blocked and therefore there is a chance of antibody side group to bind other plasma antibodies or primary/secondary antibodies when added. This would give a false MFI signal from bead if the biotinylated secondary antibody bound streptavidin. However, control wells disproved this as there was no issue concerning MFI from control wells during each of the seven 96-well plate runs.

4.4.3 Investigating bead loss and quantification of proteins during bead coating

Bio-Rad do not offer premade bead kits with antigens against PRV. Therefore, our own beads were coated, where quality and weak spots of coating had to be discussed. This included bead loss during coating, but also the quality of coating trying to specify the variables affecting the quantity of proteins coated to beads.

A total bead loss of 5 – 52 percent occurred during bead coating (fig. 3.8). Bead numbers 27 and 34 only had around half (45-52 percent) of their beads left after coating. Unequal pipetting techniques in each bead tube might explain the variation of bead loss from each bead population. Bead loss might also correlate to the magnetic separator which was used a couple of times for removal of supernatant. Even though protocol was followed, 30-60 sec in the

magnetic separator might be too little time to separate beads. Another reason for the high bead loss could also be related to the start bead count as this was only an estimated value and not a measured value at the Countess II FL. When transferring beads over to the bead tube it was important with a homogenic solution, so concentration of beads was equal in tube. Transferring beads by pipetting therefore had to be done right after beads were vortexed as beads sunk to the bottom.

1st bead 27 (ISAV FP) was a little bit aggregated when observed in Countess II FL, but still functional to use during analysis. The other beads showed no visual aggregation. The 2nd round of coated beads was not used during analysis as different protein concentrations were used during coating, since the amount of proteins available wasn't checked before starting the bead coating protocol. However, protein concentrations (21.2 µg/mL and 18.2 mg/µL) were within the recommended protein levels according to Bio-Rad`s instruction manual (5 – 12 µg protein or a concentration of 1 µg/mL – 24 µg/mL), but because of standardization they were not used in analysis.

Unfortunately, we have no methods to check the quality or quantity of antigen binding to beads. Even though no methods are available, quantification could have been possible using a monoclonal antibody that can detect proteins on beads. This has been attempted but it was not successful. Therefore, beads might be unevenly coated with proteins within same bead population and between different bead populations. However, this is unlikely as we would see a different antibody measurement (MFI) between the three runs in figure 3.7 A if this was the case.

Coating of beads can vary depending on reactions of S-NHS and EDAC to form an active ester. This is crucial as the active ester form a covalent bond between the amine group of the protein and is essential in the coupling reaction (181). The protein introduced to beads are of course of importance for the coating process, where there is a high range of protein (5 – 12 µg) that can be introduced to beads according to Bio-Rad`s instruction manual. To ensure enough protein for each active ester on bead population, the highest recommended amount of protein (12 µg) was added to beads. In addition to reaction time (2 hours for protein coating), vortex and mixing of bead tube affects the beads and solution homogenization and therefore the coating of beads. If there are carboxyl groups not bound by protein, blocking buffer was added as the last step of the bead coating process to ensure that antibodies cannot bind and give a false MFI signal.

4.4.4 Controls

Several controls were added to assay to check for errors. Firstly, the plasma was run as duplets on two different Bio-Plex 200 machines. Secondly, control beads were added to assays. Thirdly, both positive (PRV-1 infected) and negative control (uninfected group) were run at each time point. Fourthly, control wells were added for each plate during each run.

All plasma samples were run as doublets, first at NMBU and then at VI. This worked as a control to check if there were differences in measurement of antibodies binding to beads. The duplet runs also revealed that even though there is an increase in aggregation in the 2nd run and a lower bead count (down to a minimum of 10 beads per bead population), this doesn't affect the measured level of antibodies (fig. 3.6, 3.7).

Control beads ISAV FP and ISAV FP-LM detected unspecific antibody binding that were not inactivated by heat treatment. These unspecific antibodies may be produced as a result of PRV injection (polyreactive antibodies), produced due to antigens exposed to earlier, or be natural antibodies. Therefore, ISAV FP and ISAV FP-LM are important controls to check background in figure 3.1-3.4.

The negative control group worked to check for antibodies in plasma that were already there without exposure to antigen (natural antibodies) and production of antibodies towards antigens the fish had been exposed to earlier. All fish from this trial were from the same batch, and most likely exposed to the same microorganisms earlier in life. Therefore, good controls for background antibodies.

Control wells are crucial when running Bio-Plex to check that assay reagents and measurements are happening properly. Also, if unspecific background from secondary antibodies and detection reagents happens, it is an indicator for where this error happened in the protocol. Unfortunately, some of the control wells during analysis contained plasma which were not from the PRV-1 group (positive control) with the highest antibody levels. Plate 1 controls were added plasma from the InPRV-1 group which didn't show PRV specific antibody production in this assay. Plate 3 were added plasma from the PRV-2 group which also had visually low antibody levels. This weakens the assay, but during the Bio-Plex analysis none of the control wells, negative and positive controls showed any concerning results indicating that there were not any errors in the detection method.

4.5 Future perspectives

4.5.1 Protection of PRV-1 infection

What will a vaccine against HSMI mean for the future?

Mortality from HSMI usually varies from insignificant to 20 percent in farms (31). A growing world population increases the demand for food. An optimal vaccine against HSMI that could minimize mortality would therefore not only decreases suffering of fish and increases the economics of the companies. It also would increase the sustainability by ensuring that fish reach the dinner plate and contribute to three of the UN`s sustainability goals (goal 2-zero hunger, goal 8-economic growth and goal 14-life below water) (fig.4.1).

Figure 4.1. An optimal PRV-vaccine would benefit three of UNs sustainability goals. *More food would reach the dinner table decreasing hunger (goal 2) and increasing economics (goal 8) contributing to a more sustainable aquaculture industry (goal 14). An optimal PRV vaccine would decrease mortality of Atlantic salmon and therefore increase the value creation and profit by investing in fish welfare and health. Retrieved from The Global Goals.*

An optimal vaccine against HSMI should also protect against PRV-1 infection and avoid spreading PRV-1 to other fish. The vaccine should also be completely cleared from fish after injection. Fish were protected after immunization with PRV-3 and did not infect cohabs five weeks after immunization. However, PRV-3 was not completely cleared from fish at the end of the trial period, 18 wpc (162). Attenuated vaccines that are not completely cleared might cause some challenges as the fish might shed virus to the environment. The virus might then infect other species. It is especially problematic if the virus mutate and become more virulent (188). Brown trout have been found to have a high PRV-3 infection prevalence (161) and might

therefore have a higher susceptibility to the virus. It has also been speculated that PRV-3 can induce a severe disease in brown trout (189). Brown trout can therefore be infected by vaccinated farmed Atlantic salmon if not completely cleared after infection. However, there is no doubt that PRV-3 can be used to provoke specific immune responses towards PRV-1. The detection of PRV specific antibodies measured from fish immunized with PRV-3, may be one of many reasons that fish gained an efficient cross-protection against PRV-1. However, antibody levels do not necessarily correlate with the protection, which can be associated with other the long term protective immune responses (168).

The mammalian orthoreovirus (MRV) is used as a model for predicting structural and functional properties of PRV (135). Therefore, antibodies binding to σ1 are believed to be neutralizing as PRV σ1 is predicted to be the receptor binding protein. Antibody mediated protection has also shown neutralizing functions towards the MRV σ1 protein and an induced production of neutralizing antibodies when MRV σ1 protein is presented (141, 142). The PRV σ1 might be a good vaccine component in the future. This because there was a high antibody response detected towards PRV-1 σ1 from groups injected with PRV-1-3.

4.5.2 Multiplexed magnetic bead-based immunoassay for the Aquaculture industry

Bio-Plex (Luminex) assay is a sensitive bead based multiplex assay. Results can be obtained in a shorter time with less work and sample material compared to comparable analytical techniques (i.e ELISA) (177). Multiplexing has in other words great advantages to offer the growing Aquaculture industry and will be discussed in paragraphs below.

Antigens from several pathogens can be isolated and coated to beads and many antibodies screened for in the same well. This, however, is dependent on finding antigens that the immune system target, get the right coating of antigens to beads (right structure and quantity), and knowing how much binding is specific and unspecific using appropriate controls. There is a high level of unspecific antibody binding in fish and this is probably one of the biggest limitations of the Bio-Plex assay and other serological antibody assays as well. Most likely all the PRV antigens used have bound unspecific antibodies to some degree. However, if unspecific antibodies detected on control antigens are lower than antibodies detected on PRV antigens this would indicate a higher degree of specific antibodies than unspecific antibodies bound to PRV antigens.

As only small amounts of blood or mucus are needed for detection of analytes (antibodies, cytokine, chemokines, etc.), the sampling can potentially be completed with no lethal outcome. This also give the possibility to sample the same fish several times over a long period of time. Study of antibodies from one individual over a longer time frame could give important knowledge about development of how vaccines activate the adaptive humoral part of the immune system. It could also give knowledge about specific antibodies and unspecific antibodies produced before, during and after an infection of a bacteria or virus. Antibodies can also be detected after the pathogen is cleared from fish and diagnosed if fish has been infected by a pathogen. Bio-Plex might therefore be of importance for fish health biologist and veterinarians (fish or other animals) too identify exposed populations. In particular if pathogen circulates asymptomatic in fish, having an insignificant low mortality rate which is undetected by farmers, but still trigger a specific antibody response in fish.

Furthermore, one could also differentiate how an infection would affect the antibody production during different seasons when placed in sea cages with and without infection. Also, checking variables as sea cages in the south and north, age of fish or other environmental variables. This would be interesting because it would tell and prepare farmers when fish might be more susceptible to the pathogens.

A multiplex immunoassay targeting different antigens from different pathogens can therefore be used as a tool for screening and diagnostic purposes in the aquaculture industry. However, proper validation must be completed for each of the antigens before they can be used in a diagnostic tool. The aquaculture industry being one of the fastest growing industries in Norway need tools contributing to a faster diagnostics and thereby better surveillance of infectious diseases and immunity. Better surveillance of diseases and immune responses would also contribute to improved welfare and sustainability (fig. 4.1).

5.0 Conclusion

1) A primary infection with PRV-2 and PRV-3 resulted in a specific antibody response against PRV-1 in Atlantic salmon. PRV specific antibodies produced after injection with PRV-2 and PRV-3 could cross-bind PRV-1 σ1-LM.

2) PRV specific antibodies were detected from 5-10 wpc after immunization by PRV-2 and PRV-3. No antibodies were visually detected in fish injected with inactivated PRV-1. PRV specific antibodies appeared highest after PRV-1 infection.

3) After PRV-1 shedder challenge, the PRV specific antibody were detected 12-18 wpc for fish immunized with PRV-2, PRV-3 and inactivated PRV-1. The PRV specific antibody response was boosted for the inactivated PRV-1 and PRV-2 immunized group. The PRV specific antibody response decreased for PRV-3 immunized group. This reflected virus levels and protection in the PRV-3 group only.

4) After PRV-1 infection specific antibodies targeting PRV-1 σ1 were detected from 5-18 wpc and antibodies targeting PRV-1 µ1C were detected 2-18 wpc.

5) A polyreactive antibody response was detected in PRV-1 infected plasma despite heat treatment.

6) PRV-3 µNS and PRV-3 σ1 antigens did not work in this assay and could not be used as a reliable detection method of PRV specific antibodies.

7) Analysis on two different Bio-Plex machines revealed a higher aggregation in the second run and therefore a lower bead count. Bead counts as low as eight beads per bead population did not show any difference in antibody measurement (MFI). A bead count cut off on 10 beads per bead population was set as a minimum.

6.0 Appendix

6.1 Attachment: Plasma microtiter plate setup

The plasma samples consisted of a three-digit system. The first number stated the week the plasma sample was take (week post challenge), the second number stated the group/tank and the last number explained the given identification number of fish.
Example. 1014 (10 wpc, group 1, fish 4).

Table 6.1. Plate 1 - Group 1 (2, 5, 8 and 10 wpc), where C stands for cohab.

Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A	211	511	811	811C	1011	1011C						
B	212	512	812	812C	1012	1012C						
C	213	513	813	813C	1013	1013C						
D	214	514	814	814C	1014	1014C						
E	215	515	815	815C	1015	1015C						
F	216	516	816	816C	1016	1016C						
G	217	517	817		1017							
H	218	518	818		1018							

Table 6.2. Plate 2 - Group 2 (2, 5, 8, 10 wpc), where C stands for cohab.

Plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A	221	521	821	821C	1021	1021C						
B	222	522	822	822C	1022	1022C						
C	223	523	823	823C	1023	1023C						
D	224	524	824	824C	1024	1024C						
E	225	525	825	825C	1025	1025C						
F	226	526	826	826C	1026	1026C						
G	227	527	827		1027							
H	228	528	828		1028							

Table 6.3. Plate 3 - Group 3 (2, 5, 8, 10 wpc), where C stands for cohab

Plate 3	1	2	3	4	5	6	7	8	9	10	11	12
A	231	531	831	831C	1031	1031C						
B	232	532	832	832C	1032	1032C						
C	233	533	833	833C	1033	1033C						
D	234	534	834	834C	1034	1034C						
E	235	535	835	835C	1035	1035C						
F	236	536	836	836C	1036	1036C						
G	237	537	837		1037							
H	238	538	838		1038							

Table 6.4. Plate 4 - Group 4 and 5 (week 0, 2, 5, 8, 10 wpc)

Plate 4	1	2	3	4	5	6	7	8	9	10	11	12
A	1	241	251	541	551	841	851	1041	1051			
B	2	242	252	542	552	842	852	1042	1052			
C	3	243	253	543	553	843	853	1043	1053			
D	4	244	254	544	554	844	854	1044	1054			
E	5	245	255	545	555	845	855	1045	1055			
F	6	246	256	546	556	846	856	1046	1056			
G	7	247	257	547	557	847	857	1047	1057			
H	8	248	258	548	558	848	858	1048	1058			

Table 6.5. Plate 5 - Group 1, 2b, 3b, 4b, 5a, 5b (week 12 wpc)

Plate 5	1	2	3	4	5	6	7	8	9	10	11	12
A	1211	1221	1231	1241	125a1	125b1						
B	1212	1222	1232	1242	125a2	125b2						
C	1213	1223	1233	1243	125a3	125b3						
D	1214	1224	1234	1244	125a4	125b4						
E	1215	1225	1235	1245	125a5	125b5						
F	1216	1226	1236	1246	125a6	125b6						
G	1217	1227	1237	1247	125a7	125b7						
H	1218	1228	1238	1248	125a8	125b8						

Table 6.6. Plate 6 - Group 1, 2b, 3b, 4b, 5a, 5b (week 15 wpc)

Plate 6	1	2	3	4	5	6	7	8	9	10	11	12
A	1511	1521	1531	1541	155a1	155b1						
B	1512	1522	1532	1542	155a2	155b2						
C	1513	1523	1533	1543	155a3	155b3						
D	1514	1524	1534	1544	155a4	155b4						
E	1515	1525	1535	1545	155a5	155b5						
F	1516	1526	1536	1546	155a6	155b6						
G	1517	1527	1537	1547	155a7	155b7						
H	1518	1528	1538	1548	155a8	155b8						

Table 6.7. Plate 7 - Group 1, 2b, 3b, 4b, 5a, 5b (week 18 wpc)

Plate 7	1	2	3	4	5	6	7	8	9	10	11	12
A	1811	1821	1831	1841	185a1	185b1						
B	1812	1822	1832	1842	185a2	185b2						
C	1813	1823	1833	1843	185a3	185b3						
D	1814	1824	1834	1844	185a4	185b4						
E	1815	1825	1835	1845	185a5	185b5						
F	1816	1826	1836	1846	185a6	185b6						
G	1817	1827	1837	1847	185a7	185b7						
H	1818	1828	1838	1848	185a8	185b8						

6.2 Attachment: Plasma samples analysed on bio-plex (96-well plate)

96-well plate 1:

Utilized 75 of the wells in the 96-well plate, where 71 wells contained plasma samples and 4 wells worked each as control/blanks with no plasma, no primary antibody, no secondary antibody, and no streptavidin. The control wells 12F, 12G, 12H containing plasma from sample 1046, 1047, 1048, respectively (table 6.8).

Table 6.8. Overview of plasma samples on 96-well plate 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	241	251	541	551	841	851	1041	1051			
B	2	242	252	542	552	842	852	1042	1052			
C	3	243	253	543	553	843	853	1043	1053			
D	4	244	254	544	554	844	854	1044	1054			
E	5	245	255	545	555	845	855	1045	1055			
F	6	246	256	546	556	846	856	1046	1056			No pri. Ab
G	7	247	257	547	557	847	857	1047	1057			No sec. Ab
H	No plasma	248	258	548	558	848	858	1048	1058			No strepta

96-well plate 2:

Utilized 93 of the wells in the 96-well plate, where 88 wells contained plasma samples and 5 wells worked each as control/blanks with no plasma, no primary antibody, no secondary antibody, and no streptavidin. The fifth control, well 10G only contained flow buffer and streptavidin. The control wells 6G, 12G, 12H containing plasma from sample 818 (table 6.9). 50 µL of 100-folds dilution of plasma samples was made and resulted in too little plasma in all wells since all wells should have been added 50 µL (mistake). In addition, control well 12H did contain 30 µL mastermix instead of 50 µL mastermix as too little mastermix was made. Plate 2 was therefore not used in results, and plate was prepared for a second time (plate 5).

Table 6.9. Overview of plasma samples on 96-well plate 2. Not used in analysis.

	1	2	3	4	5	6	7	8	9	10	11	12
A	211	511	811	811-co	1011	1011-co	231	531	831	831-co	1031	1031-co
B	212	512	812	812-co	1012	1012-co	232	532	832	832-co	1032	1032-co
C	213	513	813	813-co	1013	1013-co	233	533	833	833-co	1033	1033-co
D	214	514	814	814-co	1014	1014-co	234	534	834	834-co	1034	1034-co
E	215	515	815	815-co	1015	1015-co	235	535	835	835-co	1035	1035-co
F	216	516	816	816-co	1016	1016-co	236	536	836	836-co	1036	1036-co
G	217	517	817		1017	No strepta	237	537	837	Flow + strepta	1037	No pri. Ab
H	218	518	818		1018		238	538	838	No plasma	1038	No sec. Ab

96-well plate 3:

Utilized 96 of the wells in the 96-well plate, where 95 wells contained plasma samples. There was one well with no plasma (blank), but no control wells as all wells were added primary antibody, secondary antibody, and streptavidin. The supposed control wells 4G, 4H, 6G containing plasma from sample 1028 (table 6.10). Control antigen ICP11 was not added and the plate was therefore prepared for a second time (plate 6).

Table 6.10. Overview of plasma samples on 96-well plate 3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	221	521	821	821-co	1021	1021-co	1211	1221	1231	1241	125a1	125b1
B	222	522	822	822-co	1022	1022-co	1212	1222	1232	1242	125a2	125b2
C	223	523	823	823-co	1023	1023-co	1213	1223	1233	1243	125a3	125b3
D	224	524	824	824-co	1024	1024-co	1214	1224	1234	1244	125a4	125b4
E	225	525	825	825-co	1025	1025-co	1215	1225	1235	1245	125a5	125b5
F	226	526	826	826-co	1026	1026-co	1216	1226	1236	1246	125a6	125b6
G	227	527	827	No pri. Ab	1027	No strepta	1217	1227	1237	1247	125a7	125b7
H	228	528	828	No sec. Ab	1028	No plasma	1218	1228	1238	1248	125a8	125b8

96-well plate 4:

Utilized 62 of the wells in the 96-well plate, where 58 wells contained plasma samples and 4 wells worked as control/blanks with no plasma, no primary antibody, no secondary antibody, and no streptavidin. The control wells 8F - 8H containing plasma from sample 1518 (table 6.11). There was made a duplicate of 10 of the wells from 1A-1H and 3A-3B to 7A-7H and 8A-8B, where mastermix 2 was added to compare 2nd coated beads with 1st coated beads to check the result and quality of coating. Plate 4 was run a second time due to low bead count number (plate 5 and 6).

Table 6.11. Overview of plasma samples on 96-well plate 4. Blue and green wells added mastermix 1 and 2, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1511	1521	1531	1541	155a1	155b1	1511	1531				
B	1512	1522	1532	1542	155a2	155b2	1512	1532				
C	1513	1523	1533	1543	155a3	155b3	1513					
D	1514	1524	1534	1544	155a4	155b4	1514					
E	1515	1525	1535	1545	155a5	155b5	1515	No plasma				
F	1516	1526	1536	1546	155a6	155b6	1516	No pri. Ab				
G	1517	1527	1537	1547	155a7	155b7	1517	No sek. Ab				
H	1518	1528	1538	1548	155a8	155b8	1518	No strepta				

96-well plate 5:

Utilized 96 of the wells in the 96-well plate, where 92 wells contained plasma samples and 4 wells worked as control/blanks with no plasma, no primary antibody, no secondary antibody, and no streptavidin. The control wells 12F - 12H containing plasma from sample 1018 (table 6.12). There was made two mastermixes, where mastermix 1 was added to control wells and column 1-8 (blue), while mastermix 2 was added to 9-11 and 12A-12D (black).

Table 6.12. Overview of plasma samples on 96-well plate 5. Blue wells added mastermix 1, green wells added mastermix 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	211	511	811	1011	231	531	831	1031	1511	152b1	153b1	155b4
B	212	512	812	1012	232	532	832	1032	1512	152b2	153b2	155b6
C	213	513	813	1013	233	533	833	1033	1513	152b3	153b3	155b7
D	214	514	814	1014	234	534	834	1034	1514	152b4	153b4	155b8
E	215	515	815	1015	235	535	835	1035	1515	152b5	153b5	No plasma
F	216	516	816	1016	236	536	836	1036	1516	152b6	153b6	No pri. Ab
G	217	517	817	1017	237	537	837	1037	1517	152b7	153b7	No sec. Ab
H	218	518	818	1018	238	538	838	1038	1518	152b8	153b8	No strepta

96-well plate 6:

Utilized 96 of the wells in the 96-well plate, where 92 wells contained plasma samples and 4 wells worked each as control/blanks with no plasma, no primary antibody, no secondary

antibody, and no streptavidin. The control wells 12B – 12D containing plasma from sample 1211 (table 6.13).

Table 6.13. Overview of plasma samples on 96-well plate 6.

	1	2	3	4	5	6	7	8	9	10	11	12
A	221	521	821	1021	1211	122b1	123b1	124b1	125a1	125b1	154b1	No plasma
B	222	522	822	1022	1212	122b2	123b2	124b2	125a2	125b2	154b2	No pri. Ab
C	223	523	823	1023	1213	122b3	123b3	124b3	125a3	125b3	154b3	No sec. Ab
D	224	524	824	1024	1214	122b4	123b4	124b4	125a4	125b4	154b4	No strepta
E	225	525	825	1025	1215	122b5	123b5	124b5	125a5	125b5	154b5	155a2
F	226	526	826	1026	1216	122b6	123b6	124b6	125a6	125b6	154b6	155a3
G	227	527	827	1027	1217	122b7	123b7	124b7	125a7	125b7	154b7	155a4
H	228	528	828	1028	1218	122b8	123b8	124b8	125a8	125b8	154b8	155a5

96-well plate 7:

Utilized 52 of the wells in the 96-well plate, where 48 wells contained plasma samples and 4 wells worked each as control/blanks with no plasma, no primary antibody, no secondary antibody, and no streptavidin (table 6.14).

Table 6.14. Overview of plasma samples on 96-well plate 7. Plate 7 was run by engineer Karen Bækken Soleim from NVI.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1811	182b1	183b1	184b1	185a1	185b1	No plasma					
B	1812	182b2	183b2	184b2	185a2	185b2	No pri. Ab					
C	1813	182b3	183b3	184b3	185a3	185b3	No sek. Ab					
D	1814	182b4	183b4	184b4	185a4	185b4	No strepta					
E	1815	182b5	183b5	184b5	185a5	185b5						
F	1816	182b6	183b6	184b6	185a6	185b6						
G	1817	182b7	183b7	184b7	185a7	185b7						
H	1818	182b8	183b8	184b8	185a8	185b8						

6.3 Attachment: Bead coating

6.3.1. Calculation of protein and PBS buffer volume:

The volume of protein and PBS (pH 7.4) buffer in bead tubes are listed in table 6.15 and 6.16.

Table 6.15. 1st coating of protein used in bio-plex analysis. Volume of protein and PBS (pH 7.4) buffer during coating. One scale (x1) coupling reaction equalled 1.25 x 10^6 beads (100 µL) with 5-12 µg protein in a volume adjusted to 500 µL by PBS buffer (pH 7.4) according to Bio-Rad`s instructions manual.

1st coating of beads:	*27*	*28*	*29*	*34*	*44*	*54*	*64*
Protein	*FP*	*FP-LM*	*ICP11-LM*	*PRV-1 µ1C*	*PRV-1 σ1-LM*	*PRV-3 σ1*	*PRV-3 µ1C*
Concentration (µg/µL)	*0.046*	*0.054*	*0.2*	*0.62*	*0.12*	*0.4*	*0.4*
Scale coupling reaction	*x1*	*x1*	*x3*	*x3*	*x3*	*x3*	*x3*
12 µg / concentration (µg/µL) x *scale coupling reaction* = **Volume of protein (µL)**	*12 / 0.046* x *1* = **261**	*12 / 0.054* x *1* = **222**	*12 / 0.2* x *3* = **180**	*12/ 0.62* x *3* = **58**	*12 / 0.12* x *3* = **300**	*12 / 0.4* x *3* = **90**	*12 / 0.4* x *3* = **90**
Total volume - *Volume of protein (µL)* = **Volume of PBS buffer (µL)**	500 - 261 = **239**	500 – 222 = **178**	1500-180 = **1320**	1500 - 58 = **1442**	1500 – 300 = **1200**	1500 – 90 = **1410**	1500 – 90 = **1410**
Total volume (µL)	*500*	*500*	*1500*	*1500*	*1500*	*1500*	*1500*

Table 6.16. 2nd coating of protein not used in bio-plex analysis. Volume of protein and PBS (pH 7.4) buffer during coating. One scale (x1) coupling reaction equalled 1.25 x 10^6 beads (100 µL) with 5-12 µg protein in a volume adjusted to 500 µL by PBS buffer (pH 7.4) according to Bio-Rad`s instructions manual.

**For bead 27 there was added 5.3 µg protein, as protein quantity was not checked before coating.*

** For bead 44 there was added 27.36 µg protein, as protein quantity was not checked before coating.*

2nd coating of beads:	*27*	*29*	*34*	*44*
Antigen	*FP*	*ICP11-LM*	*PRV-1 µ1C*	*PRV-1 σ1-LM*
Concentration (µg/µL)	*0.046*	*0.2*	*0.62*	*0.12*
Scale coupling reaction	*x0.5*	*x3*	*x3*	*x3*
12 µg / concentration (µg/µL) *x* *scale coupling reaction* = **Volume of protein (µL)**	*10.58* / 0.046* *x* *0.5* = **115**	*12 / 0.2* *x* *3* = **180**	*12/ 0.62* *x* *3* = **58**	*9.12* / 0.12* *x* *3* = **228**
Total volume - *Volume of protein (µL)* = **Volume of PBS buffer (µL)**	250 – 115 = **135**	1500 – 180 = **1320**	1500 – 58 = **1442**	1500 – 228 = **1272**
Total volume (µL)	*250*	*1500*	*1500*	*1500*

6.3.2. Calculation of End beads and Bead loss (%):

Beads at the start and end of coating with percentage of bead loss calculated in table 6.17.

Raw data illustrated in results figure 3.1.

Table 6.17. How many beads at the start, at the end and the bead loss is calculated from the concentration of coated beads from Countess II FL. The protein coating for all beads had a concentration of 0.024 µg/µL. Only 1st coated beads were used in Bio-plex analysis.

**For bead 27 there was added 5.3 µg protein (concentration of protein coating: 21.2 µg/mL), as protein quantity was not checked before coating.*

** For bead 44 there was added 27.36 µg protein (concentration of protein coating: 18.2 µg/mL), as protein quantity was not checked before coating.*

	FP (27)		FP-LM (28)	ICP11-LM (29)		PRV-1 µ1C (34)		PRV-1 σ1-LM (44)		PRV-3 σ1 (54)	PRV-3 µ1C (64)
Coating of beads	1st	2nd *	1st	1st	2nd	1st	2nd	1st	2nd *	1st	1st
How many beads (Start)	1.25 x10^6	6.25 x10^5	1.25 x10^6	3.75 x10^6	3.75 x10^6	3.75 x10^6	3.75 x10^6	3.75 x10^6	3.75 x10^6	3.75 x10^6	3.75 x10^6
How many beads (End)	6.906 x 10^5	4.230 x 10^5	8.925 x 10^5	2.775 x 10^6	2.933 x 10^6	1.785 x 10^6	2.070 x 10^6	3.525 x 10^6	2.828 x 10^6	3.555 x 10^6	3.195 x 10^6
Concentration of coated beads from countess II FL (beads/mL)	4.605 x 10^6	2.82 x 10^6	5.95 x 10^6	18.50 x 10^6	19.55 x 10^6	11.90 x 10^6	13.80 x 10^6	23.50 x 10^6	18.85 x 10^6	23.70 x 10^6	21.30 x 10^6
Total volume when measured (mL)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Bead loss (%) (End – Start) / Start * 100 %	45 %	32 %	29 %	26 %	22 %	52 %	45 %	6 %	25 %	5.2 %	15 %

6.4 Attachment: Mastermix calculations

Each sample (well) contained 2500 beads per bead population per well and the mastermix volume were 50 µL. Therefore, 2500 beads were divided with 50 which equalled 50 beads/µL.

Table 6.18. Concentration of coated beads (beads/µL) retrieved from Countess II FL – only 1st coating used in analysis.

** For bead 64, the concentration (C1) was 21 300 beads/µL on Countess II FL, but due to type error there were calculated with 21 000 beads/µL.*

**For bead 29, the concentration (C1) were 18 500 beads/µL on Countess II FL, but due to type error there were calculated with 18 000 beads/µL.*

Bead 27:	4605 beads/µL
Bead 28:	5950 beads/µL
Bead 29:	18 000* beads/µL
Bead 34:	11 900 beads/µL
Bead 44:	23 500 beads/µL
Bead 54:	23 700 beads/µL
Bead 64:	21 000* beads/µL

To find total volume of mastermix for each 96-well plate, the number of utilized wells was multiplied with 50 µL and 1.1 (10 percent extra mastermix).
To find volume of each coated beads, the dilution formula in equation 6.1 was used. This ensured same concentration (50 beads/µL) of each bead added to mastermix.

$$Volume(V1) * Concentration(C1) = Volume(V2) * Concentration(C2) \qquad Equation\ 6.1$$

C1 was the concentration of the coated beads and received by the Countess II FL (table 6.18) C2 was 50 beads/µL and V2 was total volume of mastermix (depending on utilized wells). The values were inserted to excel and solved with respect to V1.

$$V1 = \frac{V2 * C2}{C1} = \frac{Total\ volume\ of\ mastermix\ (\mu L)\ *\ 50\ beads/\ \mu L}{Concentration\ of\ coated\ bead}$$

6.5 Attachment: Primary antibody, secondary antibody, and streptavidin content/volume

Table. 6.19. Primary antibody, secondary antibody and streptavidin were added to all the 96-well plate. Both the volume and the formula for calculations are listed. Number of wells utilized can be found in appendix sec. 6.2 for all 96-well plates.

96-well plate	**1**	**2**	**3**	**4**	**5**	**6**	**7**
*Need to make (Wells utilized *50 µL*1.15):*	*4125 µL*	*5348 µL*	*5520 µL*	*3565 µL*	*5520 µL*	*5520 µL*	*2990 µL*
Primary antibody (Pri Ab) *(Need to make / 400)*	*10.3 µL*	*13.4 µL*	*13.8 µL*	*8.9 µL*	*13.8 µL*	*13.8 µL*	*7.5 µL*
Flow buffer (Need to make - pri Ab)	*4115 µL*	*5335 µL*	*5506 µL*	*3556 µL*	*5506 µL*	*5506 µL*	*2983 µL*
Secondary antibody (sec Ab) *(Need to make / 1000)*	*4.1 µL*	*5.3 µL*	*5.5 µL*	*3.6 µL*	*5.5 µL*	*5.5 µL*	*3 µL*
Flow buffer (Need to make – sec Ab)	*4121 µL*	*5343 µL*	*5515 µL*	*3561 µL*	*5515 µL*	*5515 µL*	*2987 µL*
Streptavidin *(Need to make / 50)*	*83.5 µL*	*107.0 µL*	*110.4 µL*	*71,3 µL*	*110.4 µL*	*110.4 µL*	*59.8 µL*
Flow buffer (Need to make – streptavidin)	*4043 µL*	*5241 µL*	*5410 µL*	*3494 µL*	*5410 µL*	*5410 µL*	*2930 µL*

6.6 Attachment: Raw data. Aggregation and bead count.

Comparing aggregation of beads (%) and bead count in each plasma sample (well) from group 1 (PRV-1) and group 5a (mock group) during 1st (NMBU) and 2nd (VI) run on 96 well plate 1-4.

Table. 6.20. Aggregation and bead count from group 1(PRV-1), positive control.

	First run	Second run	First run	Second run
Plasma sample	**% Agg Beads**	**% Agg Beads**	**Beads counted in well**	**Bead count in each well**
211	8	29	559	233
212	10	25	490	260
213	10	22	491	263
214	10	22	502	307
215	9	31	239	149
216	11	20	400	314
217	9	21	535	332
218	6	16	695	376
511	11	33	343	126
512	10	36	257	106
513	15	39	188	100
514	11	31	169	68
515	13	31	170	88
516	14	31	97	61
517	12	24	341	193
518	7	18	597	324
811	8	29	353	150
812	19	36	158	72
813	10	36	114	67
814	17	35	142	75
815	18	40	82	52
816	19	32	106	65
817	14	33	171	119
818	10	21	434	258
1011	10	33	288	100

1012	19	47	73	55
1013	23	54	28	9
1014	18	42	69	29
1015	19	51	46	19
1016	16	41	72	39
1017	10	31	180	91
1018	9	22	291	223
1211	10	10	820	443
1212	13	16	364	195
1213	13	20	173	107
1214	19	22	161	80
1215	15	19	252	128
1216	15	25	130	61
1217	14	18	342	177
1218	10	9	738	405
1511	9	13	944	525
1512	8	9	947	534
1513	12	10	713	423
1514	14	11	654	356
1515	15	11	506	311
1516	13	13	628	309
1517	13	15	765	419
1518	9	11	909	521

Table. 6.21. Aggregation and bead count from group 5a (Mock), negative control.

	First run	**Second run**	**First run**	**Second run**

Plasma sample	% Agg Beads	% Agg Beads	Beads counted in well	Bead count in each well
251	8	29	645	272
252	11	37	367	204
253	15	46	227	86
254	17	44	214	94
255	20	43	135	63
256	15	40	265	121
257	11	37	421	202
258	9	26	794	392
551	10	29	652	248
552	14	42	277	94
553	19	44	131	71
554	15	46	144	72
555	12	44	140	80
556	15	35	219	136
557	13	31	241	162
558	7	25	656	388
851	8	23	638	328
852	16	37	226	100
853	13	40	163	70
854	17	47	136	63
855	18	42	178	104
856	15	46	125	67
857	12	32	371	200
858	8	21	691	409
1051	8	26	645	341
1052	10	35	344	173
1053	14	37	195	101
1054	16	35	234	151

1055	14	36	211	136
1056	15	42	202	106
1057	10	25	471	261
1058	8	18	643	505
125a1	7	10	890	537
125a2	9	9	383	285
125a3	14	17	357	214
125a4	11	13	368	226
125a5	19	12	416	273
125a6	11	10	441	331
125a7	16	12	437	287
125a8	7	9	807	467
155a1	16	17	350	206
155a2	18	14	149	116
155a3	17	42	49	27
155a4	17	28	105	58
155a5	27	35	65	37
155a6	20	24	120	69
155a7	18	23	208	101
155a8	14	12	493	302

6.7 Attachment: Raw data. MFI and bead count of PRV-1 beads.

MFI and bead count from PRV-1 beads from three runs (NMBU, VI, VI) of the same 96-well plate 4.

Table. 6.22. MFI with bead count in parentheses from PRV-1 σ1-LM from group 1-4 (PRV-1, PRV-2, PRV-3, InPRV-1) for three runs. Plasma samples from 96 well plate 4 (yellow).

**** No beads counted from well.*

Plasma samples	1st	2nd	3rd
1511	24582.5 (100)	24327,0 (55)	25137,0 (15)
1512	23782.0 (89)	24681,5 (72)	23321,5 (8)
1513	20535.0 (66)	20397,5 (34)	21213,0 (6)
1514	21003.5 (60)	22328,0 (40)	22377,0 (4)
1515	19080.0 (53)	19784,0 (26)	18195,0 (3)
1516	15072.0 (51)	15344,5 (30)	16359,5 (6)
1517	10448.0 (76)	10580,5 (44)	8134,5 (6)
1518	19842.0 (89)	20997,0 (45)	21655,0 (7)
1521	6640.0 (69)	6397,0 (41)	6097,0 (3)
1522	940.0 (26)	941,0 (14)	820,0 (3)
1523	470.0 (19)	374,0 (9)	350,0 (1)
1524	6880.5 (26)	6586,0 (16)	7002,0 (6)
1525	637.0 (13)	390,0 (17)	1216,5 (4)
1526	1392.5 (22)	1009,0 (7)	777,0 (3)
1527	8083.5 (32)	5130,0 (20)	5459,0 (5)
1528	1085.0 (79)	961,0 (36)	725,0 (12)
1531	5760.0 (25)	3810,0 (6)	5719,0 (1)
1532	23957.0 (20)	24069,5 (8)	***
1533	4298.0 (7)	4389,0 (8)	***
1534	3277.5 (12)	2733,5 (12)	2315,0 (3)
1535	3763.0 (6)	3858,5 (2)	1519,0 (2)
1536	4924.0 (15)	4667,0 (10)	5489,0 (3)
1537	8168.0 (14)	8730,5 (6)	7203,0 (6)
1538	8913.5 (30)	9611,0 (38)	9025,0 (11)
1541	941.5 (42)	846,5 (28)	570,5 (4)
1542	1151.0 (23)	1050,0 (9)	964,0 (1)
1543	1274.0 (10)	996,0 (10)	2031,5 (2)
1544	1747.5 (8)	1640,0 (9)	1342,0 (1)
1545	815.0 (9)	819,5 (6)	619,0 (3)
1546	1129.0 (7)	986,0 (7)	940,0 (5)
1547	1062.0 (23)	1059,0 (9)	1115,0 (3)
1548	1796.0 (32)	1571,0 (20)	1571,5 (10)

Table. 6.23. MFI with bead count in parentheses from PRV-1 µ1C from group 1-4 (PRV-1, PRV-2, PRV-3, InPRV-1) for three runs. Plasma samples from 96 well plate 4 (yellow).

**** No beads counted from well.*

Plasma samples	1st	2nd	3rd
1511	17687.5 (208)	17586,0 (125)	18127,0 (21)
1512	15831.0 (215)	13090,0 (107)	14535,0 (34)
1513	8876.0 (171)	8489,5 (86)	9511,5 (16)
1514	16130.0 (165)	15211,0 (84)	16739,0 (5)
1515	12340.5 (114)	14642,0 (75)	13774,0 (12)
1516	12197.5 (150)	11911,0 (68)	10284,0 (10)
1517	9542.0 (165)	8831,0 (102)	8141,0 (8)
1518	7025.0 (193)	7086,0 (105)	6817,5 (8)
1521	2270.0 (163)	1962,0 (87)	1643,0 (13)
1522	262.0 (81)	198,5 (44)	189,0 (6)
1523	175.0 (57)	138,5 (28)	126,0 (5)
1524	513.0 (59)	359,0 (24)	322,0 (4)
1525	156.5 (50)	116,0 (25)	141,0 (11)
1526	635.0 (42)	435,0 (23)	356,0 (5)
1527	512.0 (69)	379,0 (39)	320,5 (14)
1528	250.0 (187)	208,0 (100)	190,0 (30)
1531	1364.0 (80)	1359,0 (37)	1367,0 (8)
1532	2356.0 (53)	1711,0 (34)	1688,0 (5)
1533	367.0 (39)	274,0 (11)	241,5 (6)
1534	1457.5 (44)	1308,0 (27)	922,0 (8)
1535	360.0 (22)	296,0 (5)	324,0 (3)
1536	893.0 (34)	630,0 (15)	515,0 (10)
1537	2335.5 (64)	1689,0 (40)	1765,0 (19)
1538	310.5 (128)	291,0 (58)	249,5 (24)
1541	489.0 (113)	456,0 (43)	430,0 (11)
1542	285.0 (39)	244,0 (28)	178,0 (4)
1543	518.5 (34)	409,0 (14)	***
1544	1015.0 (29)	885,5 (20)	720,0 (7)
1545	267.0 (19)	242,5 (14)	252,0 (3)
1546	434.0 (33)	342,5 (16)	291,0 (9)
1547	424.0 (58)	354,5 (40)	320,0 (9)
1548	299.0 (113)	232,0 (59)	230,0 (11)

6.8 Attachment: Raw data used in Bio-Plex analysis

6.8.1 Group 1 (PRV-1)

Table 6.24. Raw data from PRV-1/group 1 (positive control). Each colour represents a 96-well plate. Light grey = plate 1, dark grey = plate 3, yellow = plate 4, green = plate 5, blue = plate 6, purple = plate 7, *black = no existing samples.*

Plasma sample	PRV1 s1-LM (44)	PRV1-u1c (34)	PRV3 s1 (54)	PRV3 uNS (64)	ISAV FP (27)	ISAV FP-LM (28)	ICP11-LM (29)
1	197.0 (100)	55.0 (188)	76.0 (145)	61.0 (175)			108.0 (154)
2	281.0 (83)	74.0 (135)	116.0 (109)	74.5 (156)			218.5 (126)
3	400.0 (102)	94.0 (157)	111.0 (100)	78.5 (174)			259.0 (114)
4	255.0 (100)	72.0 (217)	106.0 (137)	72.0 (174)			155.5 (140)
5	197.5 (100)	53.0 (188)	88.0 (109)	61.0 (157)			158.0 (134)
6	237.0 (100)	59.0 (262)	87.0 (133)	54.5 (182)			221.0 (153)
7	562.0 (100)	77.0 (171)	128.0 (100)	85.0 (172)			187.5 (122)
No plasma							
211	626.0 (59)	389.5 (102)	293.0 (50)	308.0 (79)	374.5 (78)	688.0 (92)	548.0 (59)
212	343.0 (50)	219.0 (91)	153.5 (50)	163.0 (87)	153.0 (85)	245.0 (84)	362.5 (58)
213	371.0 (105)	213.5 (158)	177.0 (100)	197.0 (181)	228.5 (166)	359.0 (172)	417.5 (116)
214	722.5 (100)	514.0 (167)	286.0 (109)	388.0 (157)	421.5 (138)	782.5 (144)	926.0 (104)
215	638.5 (100)	514.5 (182)	311.0 (136)	327.0 (189)	480.0 (177)	815.0 (161)	1308.0 (132)
216	1333.0 (100)	565.5 (190)	361.0 (103)	474.5 (182)	523.0 (157)	934.0 (155)	844.5 (130)
217	3090.5 (100)	1023.0 (158)	579.0 (112)	647.0 (150)	777.0 (165)	1530.0 (169)	1286.0 (125)
218	2247.0 (100)	488.5 (176)	337.5 (106)	360.0 (163)	308.0 (138)	616.0 (179)	751.0 (118)
511	3612.0 (100)	1023.0 (149)	628.0 (105)	998.0 (172)	966.0 (168)	1698.0 (139)	1714.5 (108)
512	1812.0 (100)	765.5 (202)	413.0 (118)	781.0 (196)	680.0 (197)	1074.5 (150)	1713.0 (141)
513	1691.0 (101)	777.5 (194)	375.5 (100)	765.0 (207)	493.5 (204)	780.0 (174)	1141.5 (132)
514	3836.0 (100)	1316.5 (200)	788.0 (107)	1783.0 (209)	1169.0 (174)	1828.5 (136)	2247.5 (106)
515	1104.0 (101)	600.0 (235)	382.0 (99)	623.0 (247)	548.0 (215)	902.5 (178)	1682.0 (107)
516	2367.5 (100)	977.0 (192)	477.5 (100)	841.0 (209)	750.0 (196)	1365.0 (147)	1847.0 (114)
517	3098.5 (100)	952.5 (222)	498.5 (126)	911.0 (213)	544.0 (213)	1143.0 (164)	2095.0 (150)
518	3737.0 (100)	1011.5 (156)	536.0 (126)	759.0 (164)	617.0 (145)	1381.5 (182)	1126.0 (114)
811	24101.5 (100)	13399.0 (166)	4544.0 (133)	8593.0 (172)	5007.5 (144)	7870.0 (161)	7904.0 (130)
812	22558.0 (109)	12933.5 (206)	8543.0 (110)	8027.0 (217)	8271.0 (198)	11929.0 (125)	12621.5 (100)
813	19366.0 (111)	10733.5 (172)	3300.5 (108)	6299.0 (176)	3987.0 (193)	7076.0 (145)	6692.0 (99)
814	23713.5 (84)	5310.0 (153)	3016.0 (88)	5948.0 (182)	2964.5 (172)	6013.0 (91)	11520.0 (66)
815	23068.0 (45)	6557.0 (117)	4555.5 (58)	7245.0 (113)	4440.0 (115)	8747.0 (46)	7531.0 (39)
816	23805.0 (85)	13604.5 (180)	13138.5 (86)	12693.0 (171)	15297.5 (176)	18122.0 (133)	17036.5 (92)
817	21306.5 (100)	8366.0 (219)	4289.0 (124)	8548.0 (268)	5316.5 (222)	8785.5 (200)	6957.0 (145)
818	22022.0 (102)	9080.0 (154)	3381.5 (100)	5665.0 (169)	3709.0 (136)	7540.0 (116)	6951.0 (112)
1011	24344.0 (109)	16225.0 (197)	4064.0 (100)	11953.0 (173)	6825.0 (173)	8805.0 (174)	11472.5 (134)
1012	24879.5 (100)	17215.5 (182)	11763.5 (124)	15944.0 (200)	12504.0 (181)	14860.5 (152)	17374.0 (105)
1013	20660.5 (84)	11187.5 (166)	3930.0 (98)	9293.0 (185)	5483.5 (164)	9753.0 (132)	11739.0 (99)
1014	19788.0 (55)	12205.0 (91)	5058.0 (59)	9054.0 (99)	4353.0 (110)	8904.0 (57)	8246.5 (62)
1015	23745.0 (53)	3878.5 (100)	1790.5 (70)	6960.0 (122)	1521.5 (118)	3603.5 (58)	6517.5 (48)
1016	25637.0 (51)	12532.5 (126)	5677.0 (64)	8158.0 (107)	3995.5 (132)	7467.0 (93)	7349.5 (62)
1017	24470.0 (91)	8278.0 (229)	3330.0 (115)	8001.0 (209)	3018.0 (218)	6311.0 (171)	5464.5 (136)
1018	20763.0 (100)	8256.0 (141)	1865.5 (110)	3736.0 (157)	1472.0 (133)	3014.0 (159)	2915.0 (104)
1211	23770.0 (111)	12849.0 (172)	3848.0 (100)	6667.0 (190)	1252.0 (169)	2616.5 (142)	10538.0 (108)
1212	22886.5 (50)	6528.0 (102)	1540.5 (60)	4143.5 (114)	706.0 (77)	2251.0 (54)	3165.5 (78)
1213	22949.5 (16)	2594.0 (53)	4196.5 (30)	9338.0 (61)	1941.0 (45)	7283.0 (23)	4534.0 (26)
1214	25186.0 (17)	8350.0 (65)	2885.5 (30)	4766.0 (58)	3081.0 (32)	4706.0 (19)	6107.5 (30)
1215	25774.0 (26)	21704.0 (53)	3832.0 (27)	14415.0 (63)	2236.0 (63)	5026.0 (33)	25172.5 (32)
1216	24094.5 (18)	18349.0 (67)	4515.0 (37)	13135.0 (82)	1945.5 (30)	3837.5 (16)	10441.5 (44)
1217	24013.0 (44)	12154.5 (124)	7538.0 (73)	10472.5 (124)	3965.0 (74)	5949.0 (49)	9106.0 (77)
1218	19381.5 (106)	10592.0 (144)	4581.0 (100)	9162.5 (184)	800.0 (167)	1873.5 (116)	10008.0 (118)
1511	24304.0 (129)	15283.5 (284)	3317.0 (106)	4320.0 (233)	1676.0 (187)	3462.5 (140)	23393.5 (160)
1512	23429.0 (39)	11999.5 (112)	2663.0 (51)	6769.0 (94)	1539.0 (227)	3204.0 (153)	4142.0 (59)
1513	20726.0 (46)	5607.0 (111)	1080.0 (40)	2537.0 (89)	520.0 (165)	1073.0 (97)	1640.5 (66)
1514	21509.0 (31)	14056.0 (85)	2906.0 (33)	14504.0 (73)	1812.5 (168)	3847.0 (70)	7958.5 (52)
1515	15831.0 (35)	12261.5 (74)	1649.0 (29)	6439.5 (68)	1138.0 (136)	2232.5 (56)	2912.0 (32)
1516	20291.5 (56)	13430.0 (119)	2972.0 (41)	9369.0 (88)	1421.0 (153)	2996.0 (91)	7852.5 (54)
1517	10959.0 (69)	6587.0 (158)	1410.0 (60)	7214.0 (136)	738.0 (185)	1904.0 (101)	2752.0 (88)
1518	20405.5 (154)	6316.0 (257)	2326.0 (125)	6833.0 (248)	1121.0 (193)	2425.0 (165)	1934.0 (205)
1811	15886	3169			1389	1466	
1812	9713,5	2715			1234,5	1094	
1813	15196	8236			1469	1475	
1814	11127	4861			2720,5	3502,5	
1815	10549	4330,5			1543	1552	
1816	14394	2972,5			1511	1602	
1817	8161	4225,5			947	1095	
1818	16166	1457,5			1130,5	1163,5	

6.8.2 Group 2 (PRV-2)

Table 6.25. Raw data from PRV-2/group 2. Each colour represents a 96-well plate. Light grey = plate 1, dark grey = plate 3, yellow = plate 4, green = plate 5, blue = plate 6, purple = plate 7, black = no existing samples, orange = excluded samples due to low bead count.

Plasma sample	PRV1 s1-LM (44)	PRV1-u1c (34)	PRV3 s1 (54)	PRV3 uNS (64)	ISAV FP (27)	ISAV FP-LM (28)	ICP11-LM (29)
1	197.0 (100)	55.0 (188)	76.0 (145)	61.0 (175)			108.0 (154)
2	281.0 (83)	74.0 (135)	116.0 (109)	74.5 (156)			218.5 (126)
3	400.0 (102)	94.0 (157)	111.0 (100)	78.5 (174)			259.0 (114)
4	255.0 (100)	72.0 (217)	106.0 (137)	72.0 (174)			155.5 (140)
5	197.5 (100)	53.0 (188)	88.0 (109)	61.0 (157)			158.0 (134)
6	237.0 (100)	59.0 (262)	87.0 (133)	54.5 (182)			221.0 (153)
7	562.0 (100)	77.0 (171)	128.0 (100)	85.0 (172)			187.5 (122)
No plasma							
221	699.0 (107)	343.0 (170)	232.5 (100)	298.5 (168)	541.0 (185)	761.5 (190)	1181.0 (139)
222	546.5 (100)	387.0 (191)	231.0 (109)	363.0 (136)	903.0 (183)	1217.5 (134)	1406.0 (117)
223	578.0 (100)	459.0 (160)	286.0 (109)	428.0 (166)	610.5 (150)	1080.0 (126)	736.0 (115)
224	539.0 (100)	311.0 (193)	216.0 (105)	329.0 (182)	300.0 (239)	604.0 (154)	889.0 (144)
225	715.5 (100)	329.0 (149)	216.5 (100)	247.0 (181)	316.0 (141)	579.5 (118)	663.0 (121)
226	1245.0 (100)	648.0 (177)	314.0 (116)	415.0 (213)	674.0 (179)	1180.5 (150)	1432.5 (128)
227	651.5 (100)	534.0 (153)	262.0 (120)	360.0 (177)	477.0 (181)	835.5 (192)	710.5 (124)
228	668.5 (100)	980.0 (116)	266.0 (103)	328.5 (128)	385.0 (176)	708.5 (174)	762.0 (103)
521	7311.0 (100)	467.5 (178)	725.0 (114)	386.0 (179)	449.0 (195)	757.0 (161)	619.0 (123)
522	928.0 (100)	224.5 (186)	162.5 (100)	214.5 (200)	181.5 (180)	288.0 (132)	646.0 (122)
523	5195.0 (87)	528.0 (173)	373.0 (93)	301.5 (174)	219.0 (135)	386.0 (63)	891.0 (87)
524	6388.5 (72)	457.0 (129)	350.0 (67)	635.5 (126)	194.5 (124)	402.5 (88)	2146.5 (84)
525	4773.5 (84)	502.5 (138)	375.5 (78)	548.5 (148)	669.0 (127)	933.0 (84)	1270.0 (99)
526	7373.0 (31)	433.5 (76)	485.5 (28)	378.5 (88)	473.0 (68)	583.0 (34)	977.0 (35)
527	4660.0 (94)	801.0 (193)	436.5 (102)	470.0 (183)	304.5 (180)	671.0 (127)	1218.0 (154)
528	5470.5 (108)	462.0 (159)	355.0 (114)	540.5 (170)	259.0 (241)	409.5 (156)	1164.5 (100)
821	4077.5 (100)	210.0 (194)	255.0 (103)	420.0 (193)	488.5 (154)	709.5 (156)	873.0 (125)
822	1568.0 (69)	167.0 (156)	185.0 (94)	190.0 (173)	234.0 (137)	438.5 (76)	627.0 (89)
823	2412.0 (49)	1286.5 (144)	256.5 (54)	304.0 (115)	715.5 (92)	1097.0 (61)	1581.0 (67)
824	5385.0 (52)	187.0 (79)	300.5 (42)	497.0 (81)	242.0 (76)	427.0 (41)	554.0 (61)
825	8683.0 (36)	113.0 (72)	349.5 (32)	209.0 (73)	120.0 (91)	241.0 (45)	301.5 (36)
826	4788.0 (25)	213.0 (71)	368.0 (40)	303.5 (64)	245.0 (74)	455.0 (38)	921.0 (39)
827	10447.0 (85)	324.0 (145)	423.0 (71)	337.0 (164)	350.0 (126)	720.0 (57)	998.0 (84)
828	4027.0 (105)	422.5 (162)	377.5 (100)	380.0 (171)	607.0 (240)	979.5 (128)	1234.0 (139)
1021	606.5 (100)	161.5 (182)	340.0 (103)	274.0 (155)	228.0 (199)	364.5 (162)	905.0 (129)
1022	2732.5 (52)	175.0 (137)	206.0 (68)	301.0 (123)	128.0 (96)	155.5 (48)	439.0 (54)
1023	922.0 (42)	323.0 (65)	229.0 (47)	259.0 (65)	325.5 (54)	383.0 (29)	762.0 (41)
1024	2226.0 (22)	215.5 (72)	220.0 (32)	270.0 (48)	190.0 (61)	291.5 (28)	1227.0 (23)
1025	6687.5 (20)	232.0 (66)	309.0 (35)	271.0 (45)	176.5 (58)	347.5 (26)	621.0 (33)
1026	3127.5 (24)	368.5 (46)	233.0 (22)	363.0 (57)	162.5 (56)	286.0 (45)	584.0 (28)
1027	4911.0 (61)	692.0 (107)	568.0 (68)	608.0 (155)	526.5 (126)	998.0 (47)	1981.0 (73)
1028	2510.5 (100)	240.5 (162)	244.0 (110)	312.0 (173)	278.0 (212)	560.0 (134)	888.0 (101)
1221	8056.5 (100)	657.5 (192)	731.5 (114)	652.5 (160)	96.0 (183)	145.0 (130)	756.0 (106)
1222	1156.0 (43)	339.0 (116)	261.0 (51)	371.5 (92)	233.5 (96)	414.0 (52)	574.5 (58)
1223	2041.0 (27)	1917.5 (44)	581.0 (24)	772.5 (58)	334.5 (56)	788.5 (28)	1655.5 (22)
1224	3718.0 (20)	419.0 (69)	413.0 (31)	479.0 (52)	492.0 (27)	726.0 (22)	1083.0 (33)
1225	1451.0 (29)	1094.0 (59)	263.0 (27)	329.0 (41)	186.0 (56)	307.0 (27)	1824.0 (37)
1226	3856.0 (5)	807.0 (51)	678.5 (24)	874.0 (49)	1022.5 (34)	1484.5 (16)	2791.0 (21)
1227	2083.0 (41)	638.0 (82)	328.0 (47)	519.0 (94)	343.5 (64)	546.0 (49)	1339.0 (60)
1228	10746.5 (100)	2073.0 (163)	1036.0 (103)	1025.0 (187)	514.5 (146)	847.0 (89)	1289.0 (125)
1521	5770.0 (101)	1544.0 (188)	452.0 (82)	1140.0 (149)	286.0 (161)	523.0 (86)	2643.5 (108)
1522	1426.0 (71)	326.0 (181)	365.5 (56)	457.5 (148)	220.0 (89)	349.0 (50)	1879.0 (119)
1523	680.5 (38)	292.0 (105)	240.0 (40)	621.0 (93)	175.0 (89)	354.0 (34)	1579.5 (70)
1524	8483.0 (41)	360.5 (88)	460.0 (33)	905.0 (73)	338.0 (79)	707.0 (26)	887.0 (55)
1525	2519.0 (40)	1149.5 (126)	328.0 (33)	981.0 (121)	169.0 (67)	278.0 (25)	1548.0 (73)
1526	1633.0 (61)	807.0 (173)	636.0 (53)	842.5 (130)	540.5 (50)	1029.0 (23)	3234.0 (97)
1527	9025.0 (77)	387.0 (147)	324.0 (55)	335.0 (137)	241.0 (92)	456.0 (34)	469.5 (102)
1528	1110.0 (124)	184.0 (269)	200.0 (86)	266.0 (209)	225.0 (188)	416.0 (81)	692.0 (169)
1821	24392	5465			5670	5885,5	
1822	24349	3135			3850	3948	
1823	23108	1654,5			1739,5	2088,5	
1824	1267	304			426,5	402	
1825	1258	179,5			220	214,5	
1826	22851	675			554,5	635	
1827	23889,5	3716			2204	2577	
1828	23275	2457			4832	4248	

6.8.3 Group 3 (PRV-3)

Table 6.26. Raw data from PRV-3/group 3. Each colour represents a 96-well plate. Light grey = plate 1, dark grey = plate 3, yellow = plate 4, green = plate 5, blue = plate 6, purple = plate 7, black = no existing samples, orange = excluded samples due to low bead count

Plasma sample	PRV1 s1-LM (44)	PRV1-u1c (34)	PRV3 s1 (54)	PRV3 uNS (64)	ISAV FP (27)	ISAV FP-LM (28)	ICP11-LM (29)
1	197.0 (100)	55.0 (188)	76.0 (145)	61.0 (175)			108.0 (154)
2	281.0 (83)	74.0 (135)	116.0 (109)	74.5 (156)			218.5 (126)
3	400.0 (102)	94.0 (157)	111.0 (100)	78.5 (174)			259.0 (114)
4	255.0 (100)	72.0 (217)	106.0 (137)	72.0 (174)			155.5 (140)
5	197.5 (100)	53.0 (188)	88.0 (109)	61.0 (157)			158.0 (134)
6	237.0 (100)	59.0 (262)	87.0 (133)	54.5 (182)			221.0 (153)
7	562.0 (100)	77.0 (171)	128.0 (100)	85.0 (172)			187.5 (122)
No plasma							
231	835.5 (100)	622.0 (162)	362.5 (124)	643.0 (200)	565.0 (161)	813.0 (160)	1419.0 (125)
232	775.0 (85)	445.0 (181)	253.0 (100)	407.0 (176)	365.0 (158)	585.0 (138)	960.0 (105)
233	708.0 (43)	753.5 (88)	295.5 (46)	558.0 (98)	581.0 (103)	892.0 (67)	1606.5 (54)
234	896.5 (40)	679.5 (86)	408.5 (46)	588.0 (76)	558.5 (94)	959.0 (56)	1394.0 (55)
235	557.0 (45)	503.0 (63)	247.0 (33)	278.0 (69)	307.5 (86)	583.0 (48)	896.0 (32)
236	450.0 (57)	372.5 (114)	222.0 (61)	335.0 (113)	351.0 (131)	557.0 (105)	1572.0 (65)
237	1053.5 (72)	362.0 (167)	282.0 (69)	462.5 (170)	326.0 (152)	528.5 (110)	467.0 (120)
238	838.5 (100)	370.5 (148)	307.5 (106)	410.5 (192)	389.0 (171)	668.0 (155)	654.0 (139)
531	2250.0 (101)	658.5 (158)	481.5 (100)	819.0 (158)	634.0 (169)	978.0 (142)	2030.0 (128)
532	5538.0 (75)	1051.0 (182)	554.0 (90)	1185.0 (155)	644.0 (186)	894.0 (99)	3605.0 (107)
533	10043.5 (38)	1158.0 (95)	686.0 (59)	802.0 (69)	737.0 (87)	924.5 (66)	2476.5 (62)
534	1085.0 (57)	644.0 (108)	287.0 (47)	562.0 (131)	317.5 (124)	448.5 (72)	1266.5 (56)
535	2714.0 (32)	491.0 (92)	426.5 (38)	974.5 (90)	990.5 (106)	795.0 (59)	2458.0 (37)
536	3767.5 (50)	976.0 (107)	616.5 (58)	915.0 (106)	1127.0 (111)	1683.0 (67)	3618.5 (70)
537	5561.5 (78)	1047.0 (151)	1323.0 (81)	2000.5 (192)	1290.0 (170)	1434.0 (107)	2541.0 (90)
538	2584.0 (108)	778.0 (141)	461.0 (100)	806.0 (149)	557.0 (166)	1018.0 (135)	2404.0 (131)
831	9652.5 (100)	1028.0 (187)	994.0 (105)	1286.0 (189)	2063.0 (159)	2165.5 (140)	1874.0 (119)
832	10527.0 (63)	653.0 (98)	1056.5 (74)	1303.0 (118)	1523.0 (99)	2334.5 (88)	1642.0 (82)
833	22918.0 (39)	2226.0 (86)	3474.5 (52)	3661.0 (85)	3679.0 (84)	9611.0 (56)	4031.0 (51)
834	5834.5 (24)	502.0 (71)	679.0 (33)	1243.5 (68)	698.0 (56)	1355.0 (43)	1464.0 (37)
835	18681.0 (37)	1942.0 (68)	2464.5 (42)	4875.0 (72)	3889.0 (84)	7079.5 (60)	11860.5 (36)
836	9624.0 (27)	445.0 (53)	660.0 (27)	989.5 (52)	740.5 (44)	1514.0 (33)	1464.5 (28)
837	7739.0 (54)	319.0 (133)	440.5 (76)	711.0 (127)	468.0 (121)	844.0 (93)	832.0 (61)
838	5731.5 (100)	1200.0 (158)	942.0 (115)	1463.5 (186)	1985.0 (175)	2706.5 (142)	3220.5 (120)
1031	2980.0 (104)	383.0 (160)	477.5 (100)	700.0 (165)	642.0 (141)	925.5 (148)	1181.0 (124)
1032	10333.0 (53)	800.0 (81)	993.5 (56)	1218.5 (96)	1066.0 (92)	1818.0 (69)	2425.0 (72)
1033	14324.5 (52)	490.0 (96)	692.5 (50)	1179.0 (105)	1280.0 (96)	1860.0 (75)	1342.0 (60)
1034	4608.0 (24)	541.0 (65)	517.0 (41)	1625.5 (70)	590.5 (68)	687.0 (46)	3196.5 (30)
1035	23197.0 (19)	1656.0 (38)	2214.0 (19)	2140.0 (31)	2232.0 (43)	3336.0 (29)	2196.0 (21)
1036	8880.0 (17)	496.0 (49)	653.0 (22)	1243.0 (45)	639.0 (56)	1021.5 (26)	1025.0 (15)
1037	6500.0 (42)	2137.0 (124)	621.0 (47)	979.0 (112)	365.0 (124)	682.0 (60)	565.5 (62)
1038	16068.0 (100)	964.5 (238)	392.0 (101)	268.5 (182)	327.0 (197)	414.0 (149)	391.0 (153)
1231	5542.0 (100)	347.0 (195)	436.0 (126)	728.0 (208)	306.5 (162)	644.0 (127)	1824.0 (135)
1232	7661.5 (34)	1982.0 (115)	482.0 (46)	1373.5 (90)	431.0 (97)	1186.0 (69)	1250.0 (53)
1233	23007.5 (36)	679.5 (84)	723.0 (47)	1103.0 (61)	515.0 (62)	1282.0 (23)	2827.0 (30)
1234	21224.5 (20)	1287.0 (58)	1533.0 (23)	2057.0 (51)	269.0 (41)	557.0 (23)	1438.0 (25)
1235	4401.0 (29)	829.0 (69)	524.0 (30)	713.0 (62)	360.0 (43)	706.0 (31)	1232.0 (46)
1236	2539.0 (32)	1341.5 (24)	425.5 (24)	934.0 (34)	598.0 (63)	1111.5 (40)	2285.0 (18)
1237	7944.0 (41)	1434.0 (107)	589.5 (60)	1465.5 (84)	263.0 (73)	459.0 (40)	1575.0 (48)
1238	2570.0 (85)	389.0 (213)	347.0 (108)	732.0 (198)	333.0 (177)	592.0 (121)	1488.0 (142)
1531	7153.0 (111)	783.5 (236)	286.0 (110)	647.5 (224)	253.5 (92)	477.0 (56)	1148.5 (170)
1532	23311.0 (103)	888.0 (186)	1153.0 (104)	2389.5 (204)	489.0 (55)	1036.0 (31)	2015.0 (134)
1533	2983.5 (58)	208.0 (127)	270.0 (55)	433.5 (112)	389.0 (44)	821.0 (19)	1772.0 (73)
1534	2629.0 (78)	381.0 (217)	323.0 (64)	454.0 (148)	354.0 (37)	667.0 (17)	978.0 (119)
1535	2933.0 (82)	277.0 (153)	275.0 (80)	436.0 (175)	338.0 (17)	742.0 (5)	805.0 (113)
1536	4566.0 (73)	239.0 (185)	377.0 (97)	426.0 (156)	525.0 (51)	1089.0 (23)	568.0 (117)
1537	7820.0 (107)	683.0 (219)	434.0 (95)	539.0 (229)	515.0 (53)	883.5 (26)	2019.0 (145)
1538	9193.5 (168)	298.5 (280)	438.0 (147)	846.0 (279)	499.0 (119)	738.0 (79)	977.0 (261)
1831	2165,5	315			415,5	423,5	
1832	4316	417			539,5	480	
1833	5693	879			1075	1105	
1834	2625,5	177			257,5	241,5	
1835	3282	304			504,5	554	
1836	2550	570			539,5	606,5	
1837	3222	989			549	587	
1838	2761	369			477	453	

6.8.4 Group 4 (InPRV-1)

Table 6.27. Raw data from InPRV-1/group 4. Each colour represents a 96-well plate. Light grey = plate 1, dark grey = plate 3, yellow = plate 4, blue = plate 6, purple = plate 7, black = no existing samples, orange = excluded samples due to low bead count.

Plasma sample	PRV1 s1-LM (44)	PRV1-u1c (34)	PRV3 s1 (54)	PRV3 uNS (64)	ISAV FP (27)	ISAV FP-LM (28)	ICP11-LM (29)
1	197.0 (100)	55.0 (188)	76.0 (145)	61.0 (175)			108.0 (154)
2	281.0 (83)	74.0 (135)	116.0 (109)	74.5 (156)			218.5 (126)
3	400.0 (102)	94.0 (157)	111.0 (100)	78.5 (174)			259.0 (114)
4	255.0 (100)	72.0 (217)	106.0 (137)	72.0 (174)			155.5 (140)
5	197.5 (100)	53.0 (188)	88.0 (109)	61.0 (157)			158.0 (134)
6	237.0 (100)	59.0 (262)	87.0 (133)	54.5 (182)			221.0 (153)
7	562.0 (100)	77.0 (171)	128.0 (100)	85.0 (172)			187.5 (122)
No plasma							
241	385.0 (84)	66.5 (152)	93.5 (102)	91.0 (146)			175.0 (125)
242	374.0 (45)	57.0 (136)	90.0 (66)	76.0 (123)			100.5 (92)
243	574.0 (36)	64.0 (91)	102.0 (47)	104.0 (67)			366.0 (70)
244	577.0 (51)	77.0 (91)	120.5 (62)	93.0 (85)			141.5 (54)
245	768.5 (48)	179.0 (62)	159.0 (51)	206.0 (79)			428.0 (67)
246	414.0 (39)	75.0 (57)	103.0 (38)	100.0 (58)			224.0 (48)
247	4766.0 (63)	110.0 (121)	148.0 (75)	159.0 (119)			484.0 (73)
248	176.5 (100)	44.0 (170)	69.5 (114)	43.0 (169)			84.5 (136)
541	332.0 (100)	69.5 (202)	90.0 (110)	69.0 (161)			265.0 (126)
542	419.0 (61)	130.0 (111)	204.5 (54)	175.0 (107)			320.0 (81)
543	656.0 (23)	209.0 (42)	315.0 (29)	270.0 (52)			383.5 (48)
544	650.0 (19)	122.0 (29)	198.0 (17)	133.0 (38)			426.0 (27)
545	376.5 (12)	99.0 (21)	96.0 (6)	114.0 (12)			134.0 (29)
546	309.5 (24)	202.0 (47)	212.0 (21)	258.0 (47)			353.0 (35)
547	576.0 (47)	95.5 (72)	134.0 (66)	113.0 (83)			425.0 (71)
548	547.0 (80)	167.0 (158)	171.0 (96)	142.5 (148)			451.0 (121)
841	520.0 (77)	79.0 (183)	106.0 (82)	101.0 (125)			129.0 (97)
842	364.0 (45)	72.0 (85)	109.0 (55)	88.0 (89)			235.0 (59)
843	646.0 (13)	160.0 (31)	264.0 (15)	199.5 (30)			292.0 (16)
844	565.5 (22)	161.0 (57)	346.0 (41)	262.0 (45)			495.0 (29)
845	695.0 (19)	273.0 (33)	389.0 (13)	355.0 (27)			523.0 (21)
846	532.0 (14)	100.0 (35)	134.5 (30)	135.0 (36)			686.0 (14)
847	199.0 (34)	55.0 (62)	80.0 (50)	50.0 (53)			111.0 (43)
848	260.5 (72)	76.0 (174)	93.0 (91)	69.0 (151)			231.0 (134)
1041	442.0 (69)	100.0 (140)	132.5 (104)	106.0 (130)			579.0 (97)
1042	920.0 (43)	97.5 (52)	131.0 (33)	125.0 (73)			1050.0 (47)
1043	1474.5 (20)	244.0 (45)	220.0 (21)	194.0 (38)			301.0 (31)
1044	407.0 (20)	149.0 (42)	164.5 (34)	227.0 (41)			659.0 (28)
1045	956.0 (25)	105.0 (51)	139.0 (23)	133.0 (48)			624.0 (28)
1046	748.0 (18)	121.0 (36)	156.0 (17)	146.0 (37)			1123.0 (23)
1047	771.0 (37)	312.0 (82)	316.0 (31)	327.5 (66)			906.5 (54)
1048	703.5 (60)	90.5 (132)	149.0 (89)	97.0 (129)			300.0 (79)
1241	1298.0 (111)	343.0 (177)	183.0 (100)	232.0 (189)	148.5 (146)	210.5 (108)	583.5 (122)
1242	2583.0 (51)	254.5 (72)	923.0 (63)	527.0 (94)	203.5 (106)	423.0 (49)	3117.5 (64)
1243	2291.0 (29)	369.0 (79)	425.0 (28)	1502.0 (67)	427.5 (50)	794.0 (27)	1877.0 (48)
1244	1175.5 (22)	202.0 (34)	272.0 (24)	478.5 (48)	265.0 (29)	499.0 (16)	625.5 (22)
1245	611.0 (22)	172.0 (54)	192.0 (33)	264.0 (63)	164.0 (45)	228.0 (27)	693.0 (31)
1246	815.0 (36)	571.0 (40)	284.0 (31)	742.0 (36)	246.0 (57)	483.5 (44)	1962.0 (16)
1247	867.0 (61)	320.0 (159)	237.0 (79)	400.5 (126)	194.0 (81)	459.0 (53)	1553.0 (73)
1248	906.0 (100)	469.0 (217)	528.0 (101)	704.0 (183)	388.0 (108)	469.0 (85)	1065.0 (131)
1541	2205.0 (100)	770.5 (158)	974.0 (100)	1175.0 (163)	533.5 (136)	747.5 (50)	1682.0 (101)
1542	1273.0 (87)	322.5 (138)	337.0 (79)	935.5 (146)	308.0 (52)	539.0 (22)	1295.0 (100)
1543	1623.5 (50)	542.0 (98)	617.0 (51)	839.0 (120)	647.0 (41)	832.0 (14)	1239.0 (60)
1544	2106.0 (57)	856.5 (78)	614.0 (52)	1351.0 (89)	623.0 (47)	1581.0 (18)	2624.5 (72)
1545	787.0 (59)	269.0 (86)	269.0 (38)	433.0 (106)	353.0 (23)	510.5 (8)	771.0 (71)
1546	1144.0 (45)	384.0 (95)	518.0 (51)	812.0 (78)	780.0 (39)	1097.0 (15)	1013.0 (61)
1547	1118.0 (86)	359.0 (135)	486.0 (87)	732.0 (125)	533.0 (61)	1004.0 (29)	1822.5 (98)
1548	1912.0 (105)	468.0 (202)	394.5 (94)	457.5 (176)	258.0 (126)	443.5 (56)	758.0 (133)
1841	407,5	296			303	300	
1842	682,5	433			485,5	563,5	
1843	469	227,5			279	267	
1844	548	391,5			484	509,5	
1845	12791	1298			970	1189	
1846	11882,5	1777			1613	1823,5	
1847	14677	3120			3452	3468	
1848	12872	3864			2731	3214	

6.8.5 Group 5a (Negative group not introduced to shedders)

Table 6.28. Raw data from Mock (group 5a). Each colour represents a 96-well plate. Light grey = plate 1, dark grey = plate 3, yellow = plate 4, blue = plate 6, purple = plate 7, black = no existing samples, orange = excluded samples due to low bead count

Plasma sample	PRV1 s1-LM (44)	PRV1-u1c (34)	PRV3 s1 (54)	PRV3 uNS (64)	ISAV FP (27)	ISAV FP-LM (28)	ICP11-LM (29)
1	197.0 (100)	55.0 (188)	76.0 (145)	61.0 (175)			108.0 (154)
2	281.0 (83)	74.0 (135)	116.0 (109)	74.5 (156)			218.5 (126)
3	400.0 (102)	94.0 (157)	111.0 (100)	78.5 (174)			259.0 (114)
4	255.0 (100)	72.0 (217)	106.0 (137)	72.0 (174)			155.5 (140)
5	197.5 (100)	53.0 (188)	88.0 (109)	61.0 (157)			158.0 (134)
6	237.0 (100)	59.0 (262)	87.0 (133)	54.5 (182)			221.0 (153)
7	562.0 (100)	77.0 (171)	128.0 (100)	85.0 (172)			187.5 (122)
No plasma							
251	548.0 (100)	74.0 (165)	110.0 (111)	95.0 (164)			159.0 (105)
252	384.0 (43)	73.0 (103)	109.0 (64)	99.0 (91)			122.5 (66)
253	184.0 (29)	51.5 (52)	70.5 (30)	57.5 (64)			77.5 (52)
254	446.5 (24)	68.5 (66)	95.0 (33)	104.0 (55)			663.0 (36)
255	408.0 (9)	104.0 (59)	133.0 (15)	113.0 (34)			348.0 (18)
256	354.5 (42)	70.0 (63)	92.0 (45)	79.0 (65)			212.0 (50)
257	712.0 (61)	96.0 (91)	141.0 (64)	149.0 (129)			296.0 (76)
258	345.0 (100)	70.0 (201)	99.0 (121)	72.0 (212)			111.0 (160)
551	374.5 (72)	61.0 (199)	91.0 (96)	67.0 (155)			276.5 (130)
552	333.0 (27)	64.0 (83)	85.0 (39)	71.0 (73)			213.0 (55)
553	322.0 (11)	101.0 (46)	99.0 (12)	144.0 (34)			170.0 (28)
554	292.0 (17)	68.0 (57)	101.0 (18)	91.5 (30)			185.5 (22)
555	574.0 (21)	88.0 (44)	133.0 (27)	170.5 (28)			519.5 (20)
556	1003.0 (31)	116.0 (51)	143.0 (35)	365.0 (60)			187.0 (42)
557	431.0 (36)	93.0 (85)	92.0 (36)	93.5 (48)			300.0 (36)
558	454.0 (91)	131.0 (169)	191.0 (103)	122.0 (174)			316.0 (119)
851	453.5 (90)	68.5 (166)	112.0 (97)	100.5 (160)			306.0 (125)
852	514.0 (30)	67.5 (60)	105.5 (40)	66.0 (49)			353.0 (47)
853	311.0 (21)	62.0 (54)	95.0 (22)	69.0 (39)			202.0 (27)
854	565.0 (16)	61.0 (35)	85.0 (19)	78.0 (42)			139.0 (24)
855	552.0 (26)	118.0 (47)	113.0 (29)	105.5 (34)			220.5 (42)
856	290.0 (19)	62.0 (34)	113.0 (16)	81.0 (36)			211.5 (20)
857	191.0 (47)	53.0 (104)	84.5 (48)	65.0 (110)			227.0 (62)
858	319.0 (109)	70.0 (174)	96.0 (94)	80.0 (177)			179.0 (137)
1051	765.5 (100)	129.5 (200)	213.5 (82)	183.0 (159)			362.0 (104)
1052	532.0 (44)	106.0 (114)	110.0 (48)	93.0 (78)			283.0 (60)
1053	919.0 (23)	96.5 (50)	142.0 (38)	128.5 (52)			276.0 (32)
1054	949.0 (26)	123.0 (56)	232.5 (44)	221.0 (58)			585.0 (50)
1055	883.0 (31)	98.0 (63)	150.0 (33)	117.0 (58)			529.5 (26)
1056	643.0 (21)	79.0 (63)	126.0 (21)	126.0 (54)			385.0 (43)
1057	535.0 (63)	89.0 (155)	139.0 (64)	132.5 (110)			267.0 (79)
1058	178.0 (101)	37.0 (180)	50.0 (100)	43.0 (141)			172.0 (121)
125a1	682.5 (100)	530.5 (170)	292.0 (117)	446.5 (188)	226.0 (177)	337.0 (155)	1666.0 (108)
125a2	975.5 (44)	232.0 (141)	266.5 (62)	641.5 (104)	185.5 (96)	335.0 (51)	2081.0 (61)
125a3	5406.0 (31)	1074.0 (65)	943.0 (37)	2509.5 (62)	569.0 (81)	1416.0 (49)	2723.0 (37)
125a4	1393.0 (27)	365.0 (49)	290.5 (30)	530.0 (35)	226.0 (101)	457.0 (43)	2765.0 (31)
125a5	2142.0 (28)	325.5 (38)	467.5 (34)	512.5 (54)	434.5 (82)	975.0 (69)	1994.0 (25)
125a6	965.5 (22)	264.0 (59)	283.0 (24)	552.0 (53)	226.0 (102)	373.0 (67)	1684.0 (35)
125a7	1018.0 (48)	310.0 (155)	314.5 (76)	513.0 (129)	246.5 (108)	439.0 (69)	1205.0 (76)
125a8	635.5 (100)	153.0 (162)	190.0 (127)	260.0 (157)	171.0 (154)	283.0 (133)	439.5 (142)
155a1	398.0 (29)	161.5 (72)	181.0 (34)	322.5 (52)	211.0 (120)	380.0 (43)	
155a2	515.0 (89)	110.0 (182)	135.0 (89)	261.0 (157)	188.5 (40)	341.0 (20)	1239.0 (127)
155a3	880.5 (102)	385.0 (197)	209.0 (100)	427.0 (186)	522.0 (11)	887.5 (8)	2165.5 (128)
155a4	1066.5 (100)	299.0 (156)	296.0 (121)	459.0 (143)	200.0 (29)	279.5 (16)	2400.0 (118)
155a5	912.0 (139)	149.0 (152)	169.0 (100)	414.0 (161)	169.0 (19)	317.0 (7)	2060.0 (142)
155a6	979.0 (8)	287.0 (25)	253.5 (12)	509.0 (21)	241.5 (38)	328.5 (16)	
155a7	1208.5 (18)	462.5 (42)	349.0 (20)	855.0 (42)	353.0 (67)	977.0 (19)	
155a8	655.0 (42)	306.5 (114)	282.0 (37)	382.0 (108)	280.0 (137)	457.0 (55)	
185a1	530	401					
185a2	503,5	348					
185a3	298,5	204,5					
185a4	376	240					
185a5	422	246					
185a6	314	240					
185a7	416	253					
185a8	356	186,5					

6.8.6 Group 5b (Negative group introduced to shedders)

Table 6.29. Raw data from Mock infected with shedders 10 wpc (group 5b). Each colour represents a 96-well plate. Light grey = plate 1, dark grey = plate 3, yellow = plate 4, blue = plate 6, purple = plate 7, black = no existing samples, orange = excluded samples due to low bead count

Plasma sample	PRV1 s1-LM (44)	PRV1-s1c (34)	PRV3 s1 (54)	PRV3 sNS (64)	ISAV FP (27)	ISAV FP-LM (28)	ICP11-LM (29)
1	197.0 (100)	55.0 (188)	76.0 (145)	61.0 (175)			108.0 (154)
2	281.0 (83)	74.0 (135)	116.0 (109)	74.5 (156)			218.5 (126)
3	400.0 (102)	94.0 (157)	111.0 (100)	78.5 (174)			259.0 (114)
4	255.0 (100)	72.0 (217)	106.0 (137)	72.0 (174)			155.5 (140)
5	197.5 (100)	53.0 (188)	88.0 (109)	61.0 (157)			158.0 (134)
6	237.0 (100)	59.0 (262)	87.0 (133)	54.5 (182)			221.0 (153)
7	562.0 (100)	77.0 (171)	128.0 (100)	85.0 (172)			187.5 (122)
No plasma							
251	548.0 (100)	74.0 (165)	110.0 (111)	95.0 (164)			159.0 (105)
252	384.0 (43)	73.0 (103)	109.0 (64)	99.0 (91)			122.5 (66)
253	184.0 (29)	51.5 (52)	70.5 (30)	57.5 (64)			77.5 (52)
254	446.5 (24)	68.5 (66)	95.0 (33)	104.0 (55)			663.0 (36)
255	408.0 (9)	104.0 (59)	133.0 (15)	113.0 (34)			348.0 (18)
256	354.5 (42)	70.0 (63)	92.0 (45)	79.0 (65)			212.0 (50)
257	712.0 (61)	96.0 (91)	141.0 (64)	149.0 (129)			296.0 (76)
258	345.0 (100)	70.0 (201)	99.0 (121)	72.0 (212)			111.0 (160)
551	374.5 (72)	61.0 (199)	91.0 (96)	67.0 (155)			276.5 (130)
552	333.0 (27)	64.0 (83)	85.0 (39)	71.0 (73)			213.0 (55)
553	322.0 (11)	101.0 (46)	99.0 (12)	144.0 (34)			170.0 (28)
554	292.0 (17)	68.0 (57)	101.0 (18)	91.5 (30)			185.5 (22)
555	574.0 (21)	88.0 (44)	133.0 (27)	170.5 (28)			519.5 (20)
556	1003.0 (31)	116.0 (51)	143.0 (35)	365.0 (60)			187.0 (42)
557	431.0 (36)	93.0 (85)	92.0 (36)	93.5 (48)			300.0 (36)
558	454.0 (91)	131.0 (169)	191.0 (103)	122.0 (174)			316.0 (119)
851	453.5 (90)	68.5 (166)	112.0 (97)	100.5 (160)			306.0 (125)
852	514.0 (30)	67.5 (60)	105.5 (40)	66.0 (49)			353.0 (47)
853	311.0 (21)	62.0 (54)	95.0 (22)	69.0 (39)			202.0 (27)
854	565.0 (16)	61.0 (35)	85.0 (19)	78.0 (42)			139.0 (24)
855	552.0 (26)	118.0 (47)	113.0 (29)	105.5 (34)			220.5 (42)
856	290.0 (19)	62.0 (34)	113.0 (16)	81.0 (36)			211.5 (20)
857	191.0 (47)	53.0 (104)	84.5 (48)	65.0 (110)			227.0 (62)
858	319.0 (109)	70.0 (174)	96.0 (94)	80.0 (177)			179.0 (137)
1051	765.5 (100)	129.5 (200)	213.5 (82)	183.0 (159)			362.0 (104)
1052	532.0 (44)	106.0 (114)	110.0 (48)	93.0 (78)			283.0 (60)
1053	919.0 (23)	96.5 (50)	142.0 (38)	128.5 (52)			276.0 (32)
1054	949.0 (26)	123.0 (56)	232.5 (44)	221.0 (58)			585.0 (50)
1055	883.0 (31)	98.0 (63)	150.0 (33)	117.0 (58)			529.5 (26)
1056	643.0 (21)	79.0 (63)	126.0 (21)	126.0 (54)			385.0 (43)
1057	535.0 (63)	89.0 (155)	139.0 (64)	132.5 (110)			267.0 (79)
1058	178.0 (101)	37.0 (180)	50.0 (100)	43.0 (141)			172.0 (121)
125b1	1002.0 (100)	145.0 (185)	212.0 (125)	293.0 (177)	161.0 (229)	610.0 (187)	1211.5 (122)
125b2	1589.5 (50)	250.5 (100)	342.0 (81)	527.0 (119)	195.0 (199)	343.0 (143)	892.0 (71)
125b3	411.0 (35)	280.5 (74)	247.5 (42)	353.0 (75)	158.0 (169)	195.0 (114)	1184.0 (39)
125b4	591.5 (78)	357.5 (28)	248.5 (64)	368.0 (34)	466.0 (149)	561.0 (104)	1113.0 (22)
125b5	2240.0 (44)	316.0 (82)	596.0 (31)	576.0 (57)	306.0 (195)	531.0 (109)	3095.0 (31)
125b6	750.0 (35)	387.0 (75)	420.0 (40)	326.0 (65)	287.0 (144)	411.0 (84)	1421.0 (45)
125b7	941.0 (55)	230.0 (83)	271.0 (67)	493.5 (98)	213.0 (185)	313.0 (141)	1515.0 (59)
125b8	1300.0 (90)	254.5 (146)	269.0 (101)	416.0 (153)	219.0 (196)	279.0 (153)	2048.0 (113)
155b1	1088.0 (21)	332.5 (70)	273.0 (34)	653.0 (72)	326.0 (95)	525.5 (30)	
155b2	973.0 (15)	317.0 (21)	316.0 (12)	390.5 (30)	300.5 (34)	404.5 (12)	
155b3	2490.0 (3)	876.0 (7)	549.5 (4)	1983.0 (9)	491.5 (4)	1267.5 (6)	
155b4	1218.0 (108)	700.5 (188)	886.0 (77)	1432.0 (133)	1136.0 (18)	2494.0 (12)	2791.0 (157)
155b5	2278.0 (5)	836.0 (18)	1061.0 (11)	2268.0 (8)	758.5 (26)	1787.0 (11)	
155b6	870.0 (97)	203.0 (247)	231.0 (86)	486.0 (189)	247.0 (17)	287.0 (5)	2448.0 (148)
155b7	570.0 (126)	341.5 (218)	263.5 (100)	484.5 (180)	490.5 (34)	869.0 (11)	1093.0 (158)
155b8	600.0 (118)	245.0 (251)	213.0 (117)	353.0 (211)	254.0 (89)	552.0 (41)	631.0 (177)
185b1	14038	5962					
185b2	13458	3653					
185b3	19485	3006					
185b4	13191	2658					
185b5	20030	3835					
185b6	22774	4072,5					
185b7	15215	3906					
185b8	12403	2131					

6.9 Attachment: Paper

Piscine Orthoreovirus (PRV)-3, but Not PRV-2, Cross-Protects against PRV-1 and Heart and Skeletal Muscle Inflammation in Atlantic Salmon

Article *in* Vaccines · March 2021

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Piscine Orthoreovirus (PRV)-3, but Not PRV-2, Cross-Protects against PRV-1 and Heart and Skeletal Muscle Inflammation in Atlantic Salmon

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Abstract: Heart and skeletal muscle inflammation (HSMI), caused by infection with *Piscine orthoreovirus-1* (PRV-1), is a common disease in farmed Atlantic salmon (*Salmo salar*). Both an inactivated whole virus vaccine and a DNA vaccine have previously been tested experimentally against HSMI and demonstrated to give partial but not full protection. To understand the mechanisms involved in protection against HSMI and evaluate the potential of live attenuated vaccine strategies, we set up a cross-protection experiment using PRV genotypes not associated with disease development in Atlantic salmon. The three known genotypes of PRV differ in their preference of salmonid host species. The main target species for PRV-1 is Atlantic salmon. Coho salmon (*Oncorhynchus kisutch*) is the target species for PRV-2, where the infection may induce erythrocytic inclusion body syndrome (EIBS). PRV-3 is associated with heart pathology and anemia in rainbow trout, but brown trout (*S. trutta*) is the likely natural main host species. Here, we tested if primary infection with PRV-2 or PRV-3 in Atlantic salmon could induce protection against secondary PRV-1 infection, in comparison with an adjuvanted, inactivated PRV-1 vaccine. Viral kinetics, production of cross-reactive antibodies, and protection against HSMI were studied. PRV-3, and to a low extent PRV-2, induced antibodies cross-reacting with the PRV-1 σ1 protein, whereas no specific antibodies were detected after vaccination with inactivated PRV-1. Ten weeks after immunization, the fish were challenged through cohabitation with PRV-1-infected shedder fish. A primary PRV-3 infection completely blocked PRV-1 infection, while PRV-2 only reduced PRV-1 infection levels and the severity of HSMI pathology in a few individuals. This study indicates that infection with non-pathogenic, replicating PRV could be a future strategy to protect farmed salmon from HSMI.

Keywords: heart and skeletal muscle inflammation; *Piscine orthoreovirus*; vaccine; atlantic salmon; antibodies; immune response

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1. Introduction

Infections represent a constant challenge and threat against fish health and welfare in aquaculture. Modern farming of Atlantic salmon (*Salmo salar*) is characterized by high-density populations, rapid growth, short production cycles, and artificial adaptation to

sea water. This life cycle does not ensure natural pathogen exposure in early life or the natural training of the fish innate immune system [1]. When transferred to the sea, the untrained immune system may not be ready to handle the novel repertoire of pathogens. High-density populations increase infection pressure, and transportation and handling procedures increase disease susceptibility due to stress [2]. In Atlantic salmon aquaculture, vaccines have been effective in protecting the fish from many diseases, but several viral diseases remain unsolved challenges [3]. One of the viral diseases of concern in European Atlantic salmon aquaculture is heart and skeletal muscle inflammation (HSMI) caused by *Piscine orthoreovirus* (PRV) [4,5].

PRV particles are non-enveloped with a double-layered protein capsid and a segmented double-stranded RNA genome [6]. PRV is a common virus infection in salmonids, and PRV-1 is the genotype associated with HSMI in farmed Atlantic salmon [5,7]. PRV is ubiquitous in the sea water phase of salmonid aquaculture [8] and is also emerging in fresh water facilities. However, PRV-1 is found to a lower extent in salmonids in the wild [9,10]. PRV-1 was first described in 2010 [4], whereas HSMI emerged in Norway and Scotland a decade earlier [11,12]. The causality between PRV-1 and HSMI was proven experimentally in 2017 using highly purified virus to induce disease [5]. PRV-1 is proposed to infect Atlantic salmon via the intestinal tract [13], followed by a massive infection of red blood cells and high plasma viremia [14,15]. Following the peak infection in red blood cells, the virus infects cardiomyocytes, which may result in an inflammatory response dominated by cytotoxic T-cells in the heart [16,17]. This inflammatory response is a hallmark of HSMI. In Atlantic salmon populations, the disease usually gives a moderate mortality that in severe cases may accumulate to 20% [11]. The relative high frequency of outbreaks makes HSMI a significant problem for the salmon farming industry. The PRV-1 infection becomes persistent in Atlantic salmon, and based on PRV prevalence in farm escapees [10], near 90% of Norwegian farmed salmon are PRV-infected in the marine phase, while near 100% of a small number of escaped Atlantic salmon were reported infected in Washington and British Columbia [18]. The long-term effects of PRV-1 infection are disputed, but the virus has been associated with the worsening of black spots in the skeletal muscle [19], a significant quality problem for the salmon production industry. This association is, however, disputed [20]. PRV-1 is also found in Canadian aquaculture, but few cases of HSMI have been reported [21], and HSMI has not been reproduced experimentally using Canadian isolates [22–24]. Different PRV-1 isolates with genetic variation have been shown to differ in the ability to induce HSMI [7]. PRV-1 has also been reported to infect other salmonid species [25].

Two additional genotypes of PRV, PRV-2 and PRV-3, have been described. They both infect salmonids, but with a different ability to infect and cause disease in the various salmonid species. PRV-2 infects coho salmon (*Oncorhynchus kisutch*) in Japan, causing erythrocytic inclusion body syndrome (EIBS) [26]. The main host species of PRV-3 may be wild brown trout (*S. trutta*) [27], but disease has only been found in farmed rainbow trout (*O. mykiss*), where PRV-3 is associated with heart inflammation and anemia [28–30]. Nucleotide alignment shows 80% (PRV-2) and 89% (PRV-3) identity to PRV-1 [31]. PRV-3 has previously been shown to infect Atlantic salmon experimentally, but without inducing HSMI [29]. Current information on PRV subtypes and distribution was recently reviewed [32].

No vaccines have been marketed against HSMI, but two different experimental vaccination approaches have been published. An inactivated whole virus vaccine, based on purified virus, was shown to give partial protection against HSMI, but less efficient protection against infection and virus replication [33]. Although promising, this approach has been hampered by the problem of producing PRV-1 for vaccine development, as no cell lines efficiently produce viral progeny [34]. A DNA vaccine approach has also been tested, and partial protection against HSMI was reported for a vaccine combining non-structural PRV-1 proteins with outer capsid antigens [35]. Although with some protective effects against HSMI, none of these vaccines have been able to block PRV-1 infection.

PRV-1 infection has been reported to induce strong innate antiviral responses in infected red blood cells [36]. Expression analysis of adaptive immune response genes has indicated that both humoral and cellular responses are induced [37], and it has been shown that infected fish produce specific antibodies against the outer capsid spike protein σ1 [38], predicted to be the receptor-binding protein [39]. The cellular immune response initiated by PRV-1 in Atlantic salmon is strongly associated with HSMI development, and the typical HSMI myocarditis is dominated by an influx of cytotoxic T-cells [16,17]. However, this response is also associated with virus eradication from heart tissue, making cellular immunity a two-edged sword in HSMI [16,40].

The purpose of this study was to determine if PRV-2 or PRV-3 infection in Atlantic salmon could provide protection against a consecutive PRV-1 infection and HSMI. We compared the protection induced by PRV-2 and PRV-3 to an inactivated PRV-1 vaccine, and characterized immune responses, including the production of cross-reactive PRV-specific antibodies. The results show that PRV-3 infection in Atlantic salmon, in contrast to PRV-2, blocks a secondary infection with PRV-1, and that cross-protective antibodies may be one of the mechanism involved.

2. Materials and Methods

2.1. Experimental Trial and Sampling

The trial was performed at the Aquaculture Research station at Kårvika, Troms, Norway, approved by the Norwegian Animal Research Authority, and performed in accordance with the recommendations of the current animal welfare regulations: FOR-1996-01-15-23 (Norway).

The PRV-1 infection material was prepared from two frozen blood cell pellets (−80 °C) with PRV-1 qPCR ct values of 17.6 and 16.4, harvested from a PRV-1-infected Atlantic salmon from a previous experimental trial [5]. The virus isolate (PRV-1 NOR2012-V3621 [5]) originated from an HSMI outbreak in mid-Norway in 2012 and had been passaged in prior experimental trials, all resulting in HSMI. The PRV-3 infection material was a blood pellet that originated from a Norwegian outbreak in 2014 (PRV-3 NOR2014, [28]) and has been passaged twice experimentally in rainbow trout [30]. The mock-blood cell lysate originated from control fish from an Atlantic salmon experimental trial. The blood cell lysate from PRV-1, PRV-3 and mock was prepared by diluting the blood pellet (plasma removed prior to freezing) 1:10 in L15-medium, sonicating five times at 20 kHz for 10 s with 1 min rest in between and centrifuging at 3000× *g* for 10 min before the collection of the supernatant. The PRV-2 infection material (PRV-2, [26]) originated from a frozen spleen sample from a Coho salmon. The tissue sample was homogenized in L15 medium as described for the blood pellets. The inactivated PRV-1 material was prepared from a batch of purified PRV-1 particles (PRV-1 NOR2012, 5.35 × 10^9 copies /mL) by PHARMAQ AS, as described in a previously published trial [33]. In short, the batch was formalin-inactivated and prepared as a water-in-oil formulation where the water phase (containing PRV antigens) was dispersed into a mineral oil continuous phase containing emulsifiers and stabilizers.

At the start of the trial, a total of 630 fish (*Salmo salar* L) were divided into four experimental groups of 75 fish and one mock control group of 125 fish, while 190 naïve fish from the same group were kept for use as transmission controls and future virus shedders. The experimental fish were kept in freshwater (10 °C, 24:0 light:dark cycle, >90% O_2) and injected intraperitoneally (ip) with 0.2 mL of immunization material described above. Eight fish were sampled prior to Injection Week 0, and from each of the five experimental groups Week 2 and 5. Five weeks after the start of the experiment, 12 naïve fish labelled by tattoo pen were added to each of the tanks containing fish infected with PRV-1, PRV-2 and PRV-3 to monitor transmission of virus. At Weeks 8 and 10, eight experimental fish and six transmission control fish were sampled from each of these groups. At Week 8, 140 naïve fish in a separate tank were injected ip with 0.2 mL of a newly prepared batch of PRV-1 blood cell lysate (PRV-1 NOR2012, same origin and preparation method) and left for two weeks. After Sampling Week 10, 35 fish remained in each of the experimental groups, and

70 fish in the mock-infected control group. The mock group fish were divided into two tanks of 35 fish each, and three experimental tanks (PRV-2, PRV-3, InactPRV-1) and one of the mock-tanks were added to an equal number (35) of tattoo-labelled pre-infected PRV-1 shedder fish. No shedders were added to the original PRV-1 tank, and the other mock group was kept as a negative control. The number of tanks included in the experiment was now 6, and eight fish from each group were sampled on Weeks 12, 15 and 18. No fish died during the experiment.

At each sampling, blood was drawn from the caudal vein on BD Medical Vacutainer heparin-coated tubes (BD Medical, Mississauga, ON, USA). Hearts were sampled on 10% formalin for histology and samples from the heart tip and spleen were sampled on 0.5 mL of RNALater (Qiagen, Hilden, Germany) in separate bar-coded microtubes (FluidX Ltd., Manchester, UK) along with additional organ samples not analyzed here. Blood samples were stored at 4 °C for a maximum of 6 h, centrifuged (3000× g for 5 min at 4 °C), and plasma and cell pellets were separated into different microtubes and stored at −80 °C. RNALater samples were stored at 4 °C for 24 h followed by freezing at −20 °C. Formalin samples were stored at RT for 24 h, after which formalin was changed to 70% ethanol, and thereafter stored cold (4 °C).

2.2. RNA Preparation and RT-qPCR for Virus and Host Response Gene Analyses

Tissue samples from the spleen and heart (25 mg) on RNALater (Qiagen) were transferred to 0.65 mL Qiazol lysis reagent (Qiagen) with a 5 mm steel bead and homogenized in a TissueLyzer II (Qiagen) for 2 × 5 min at 25 Hz followed by chloroform inclusion, and the aqueous phase was collected. RNeasy Mini QIAcube Kit (Qiagen) was used as per the manufacturer guidelines for automated RNA isolation. RNA concentrations were quantified using the Nanodrop ND-100 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA was eluted in RNase-free water and stored at −80 °C until further use. For the PRV subtype expression analysis, i.e., PRV-1 and PRV-3, one-step RT-qPCR was performed using an Agilent Brilliant III Ultra Fast kit (Agilent Technologies, Santa Clara, CA, USA) with 100 ng (5 µL of 20 ng/µL) RNA per reaction in duplicates of 15 µL total reaction volume. The template was previously denatured at 95 °C for 5 min. Cycling parameters were set to 10 min for 50 °C, 3 min at 95 °C, and 40 cycles for 5 s at 95 °C and 10 s at 60 °C. The cut-off value was set to 35 and samples were run with positive and no template controls (NTC). For PRV-2 expression analysis, a Quantitect SYBR Green (Qiagen) RT-qPCR kit (catalogue number 204243) was used according to manufacturer instructions. A total of 100 ng RNA with prior denaturation at 95 °C for 5 min was used in duplicates in 15 µL of total reaction volume. Thermal conditions were 50 °C for 30 min, 95 °C for 15 min, and 40 cycles with 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Specificity of the assay was confirmed by melting curve analysis. The same threshold level and positive controls were used together with NTCs. Probes and primer sequences are given in Supplementary Table S1.

For Immune gene expression, 400 ng total spleen RNA per sample was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit with gDNA wipeout buffer (Qiagen). For qPCR, cDNA corresponding to 5 ng RNA was analyzed with Sso Advanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and 10 pmol of forward and reverse target-specific primers in a 10 µL volume in duplicate wells on a 384 well plate. The amplification program (15 s 95 °C, 30 s 60 °C) was run for 40 cycles in a CFX Touch Real-Time PCR Detection System (Bio-Rad), followed by a melt point analysis. The results were analyzed using the software CFX Manager, version 3.1.1621.0826. The expression cycle threshold level was normalized against the elongation factor (EF) 1α reference gene (ΔCt). The ΔΔCt method was used to calculate relative expression levels and fold induction compared to samples from the uninfected control samples.

2.3. Bead-Based Immunoassay

MagPlex®-C Microspheres (Luminex Corp., Austin, TX, USA) #12, #21, #27, #29, #34, #36, #44, #62 and #64 were coated with antigens using the Bio-Plex Amine Coupling Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The N-Hydroxysulfosuccinimide sodium salt and N-(3-Dimethylaminopropyl)-N′-ethylcarbod used for the coupling reaction were from Sigma-Aldrich. For each coupling reaction, 6-24 µg of recombinant protein was used. The proteins used were recombinant PRV µ1l [41], lipid-modified PRV σ1 (LM-PRVσ1), unmodified infectious salmon anemia virus fusion protein (ISAV-FP), and lipid-modified ISAV-FP (LM-ISAV-FP) [39]. The bead concentrations were determined using the Countess automated cell counter (Invitrogen, Carlsbad, CA, USA). Coupled beads were stored in black Eppendorf tubes at 4 °C for up to 10 weeks. Incubations were performed at room temperature and protected from light on a HulaMixer rotator (Thermo Fisher Scientific) at 15 rpm.

The immunoassay was performed as described earlier (8). Briefly, Bio-Plex Pro™ Flat Bottom Plates (Bio-Rad) were used. Beads were diluted in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) (Bio-Rad Diagnostics GmbH, Dreieich, Germany) and 0.05% azide (Merck, Darmstadt, Germany) (PBS+) and 2500 beads of each bead number were added to each well. AntiSalmonid-IgH monoclonal antibody (clone IPA5F12, Cedarlane, Burlington, ON, Canada) diluted 1:400 in PBS+ was used as an unconjugated anti-IgM heavy chain monoclonal antibody. Biotinylated goat AntiMouse IgG2a antibody (Southern Biotechnology Association, Birmingham, AL, USA) diluted 1:1000 in PBS+ was used as a secondary antibody, and Streptavidin-PE (Invitrogen) diluted 1:50 in PBS+ was used as the reporter flourochrome. Plates were read using two different Bio-Plex 200 (Bio-Rad) machines as part of a validation plan. The DD-gate was set to 5000–25,000, and between 20 and 100 beads from each population were read from each well. The reading was carried out using a low (standard) photomultiplier tube (PMT) setting. The results were analyzed using the Bio-Plex Manager 5.0 and 6.1 (Bio-Rad). All samples were analyzed in duplets on each of the two different Bio-Plex 200 (Bio-Rad) machines. The data used originated from one machine, but no differences were observed during validation. The data were given in mean fluorescence intensity (MFI), based on secondary antibody binding to beads, and were corrected for binding to control beads without antigen: MFI (antigen-containing beads) − MFI (control beads) = MFI (sample data).

2.4. Histopathology

Formalin-fixed hearts were paraffin embedded and routinely processed. The sections, 3–4 µm, were stained with hematoxylin and eosin (H&E, Merck, Kenilworth, NJ, USA) and studied under microscope. The slides from Experimental Weeks 15 and 18 (n = 96) were blinded to the study groups and scored by an experienced fish pathologist using a visual analogue scale from 0 to 3 as previously described [11].

2.5. Statistical Analysis

All statistical analyses were performed within GraphPad Prism 8.1.1 (GraphPad Software Inc., La Jolla, CA, USA). Ct values of the target groups (PRV-2, PRV-3 and Inact. PRV-1-injected fish exposed to PRV-1 shedder fish at 10 weeks post injection) were compared to the PRV-1 control group by using the non-parametric Mann–Whitney test due to the small sample size (n = 8) in each group. p-values of $p \leq 0.05$ were considered as significant.

3. Results

3.1. PRV Immunization Trial

The trial was performed as outlined in Figure 1. Initially (Week 0), Atlantic salmon with a mean weight of 41.3 g (+/− 5.8 g) were grouped and injected intraperitoneally (ip) with cell or tissue lysates containing infective PRV-1, PRV-2 or PRV-3, uninfected blood lysate (mock), or purified, inactivated and adjuvanted PRV-1 [33]. At 10 weeks, the mean weight of the injected fish was 107.6 g (+/− 18.4 g) with no significant difference

between groups (Supplementary file S2). At this timepoint, PRV-1-infected shedder fish were added to the remaining fish in the groups injected with PRV-2, PRV-3, inactivated PRV and half of the mock group to test the effects of immunization. Neither the initial ip challenge/immunization nor the cohabitant challenge led to mortality in any of the treatment groups, and there was no loss of fish or aberrant clinical observations during the experimental period. At the end of the experiment in Week 18, the fish mean weight was 193.6 g (+/− 29.5 g), with no statistically significant difference between groups.

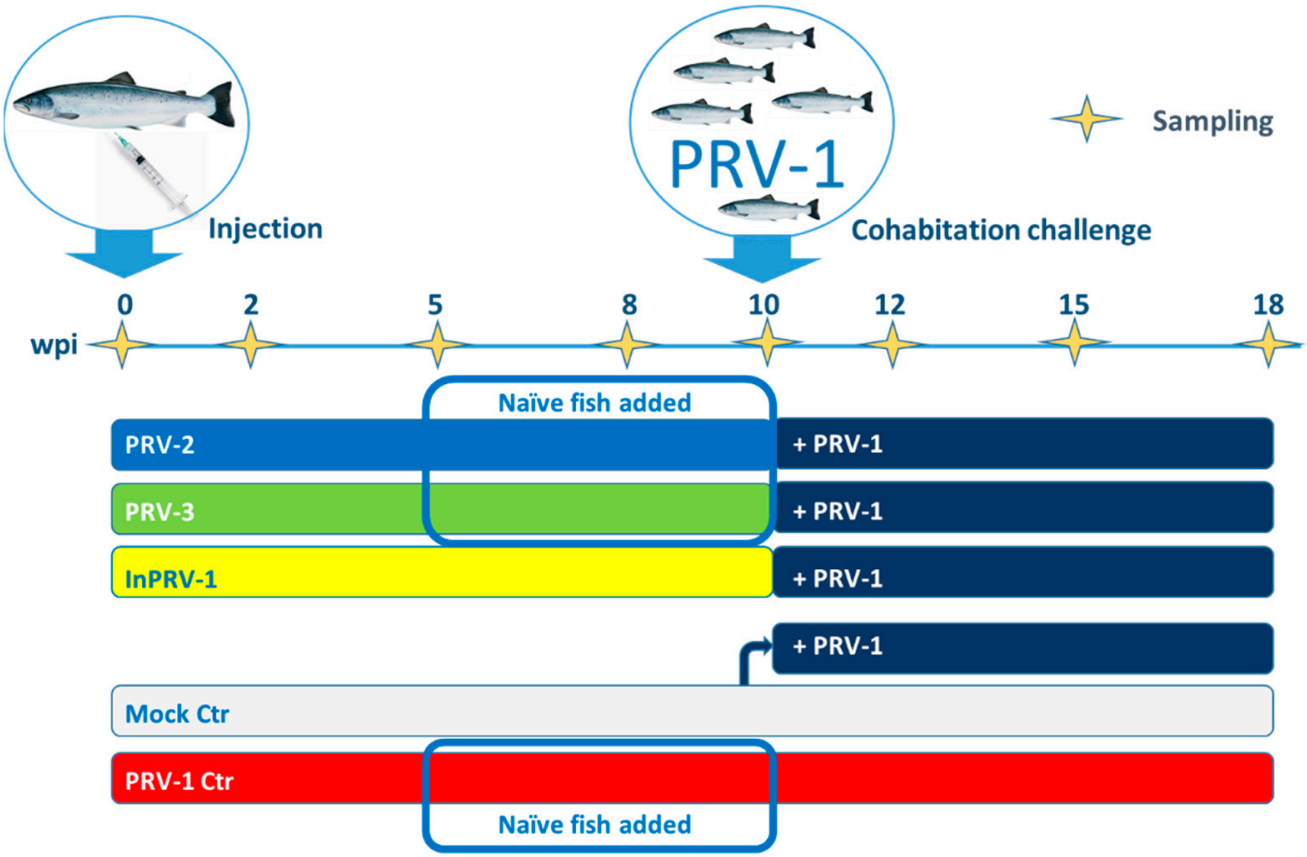


Figure 1. Groups and timeline of the *Piscine orthoreovirus* (PRV) immunization trial. Fish were immunized intraperitoneally (ip) with either spleen homogenate containing PRV-2 (blue group), blood cell lysate containing PRV-3 (green group), or purified, inactivated and adjuvanted PRV-1 (InPRV-1, yellow group). The negative control group (mock, white) was injected with blood cell lysate from uninfected fish. A positive control group was injected with PRV-1 (red). Naïve fish were added to tanks containing fish injected with infective PRV-1, PRV-2 and PRV-3 five weeks post injection (wpi) and sampled Week Eight and Ten to control viral shedding. After 10 weeks, the immunized group and half of the mock group was infected through cohabitation with fish experimentally infected with PRV-1 (shedders, dark blue) and thereafter monitored until Week 18. Yellow stars on the timeline show sampling time points (all groups).

3.2. Replication and Transmission of PRV Genotypes in Atlantic Salmon

The RNA loads of PRV-1, PRV-2 and PRV-3 were monitored by the RT-qPCR of spleen samples through the experimental period (Figure 2, Supplementary file S2). The spleen was chosen for analysis since PRV replicates in red blood cells, and spleen has been shown to reflect the levels of PRV infection in blood [42] better than, e.g., kidney. PRV-1 showed maximum replication during the first 5 weeks, as expected from previous trials (median Ct 14.79, interquartile range (IQR) Ct 14.12–15.37 (Figure 2A)), and persisted in spleen through the 18 weeks of the study with median PRV-1 levels above a Ct level of 20 at all sampling time points. Five weeks after injection, naïve fish were added to tanks of fish injected with PRV-1, PRV-2 and PRV-3 to study the transmission of the injected virus. The

naïve cohabitants added to the PRV-1 group at Week 5 were all infected 3 and 5 weeks later (Experimental Week 8 and 10, not analyzed at later time points). PRV-2 levels were generally low and reached the highest level after 2 weeks (median Ct of 26.7, IQR Ct 25.99–27.08), after which the infection declined. After 18 weeks, PRV-2 was detected in only one out of eight sampled fish. No naïve cohabitants added to the PRV-2 tank Week 5 were infected (Figure 2B). PRV-3 levels increased up to Week 5 (median Ct of 19.19, IQR Ct 18.02–20.75), then declined until Week 18 (Figure 2C). The added naïve cohabitants were not infected. No cross-infection was observed between the tanks, and no replication was observed in the fish injected with inactivated PRV-1, as monitored on Weeks 2, 5 and 10 (Supplementary Figure S1, Supplementary File S2).

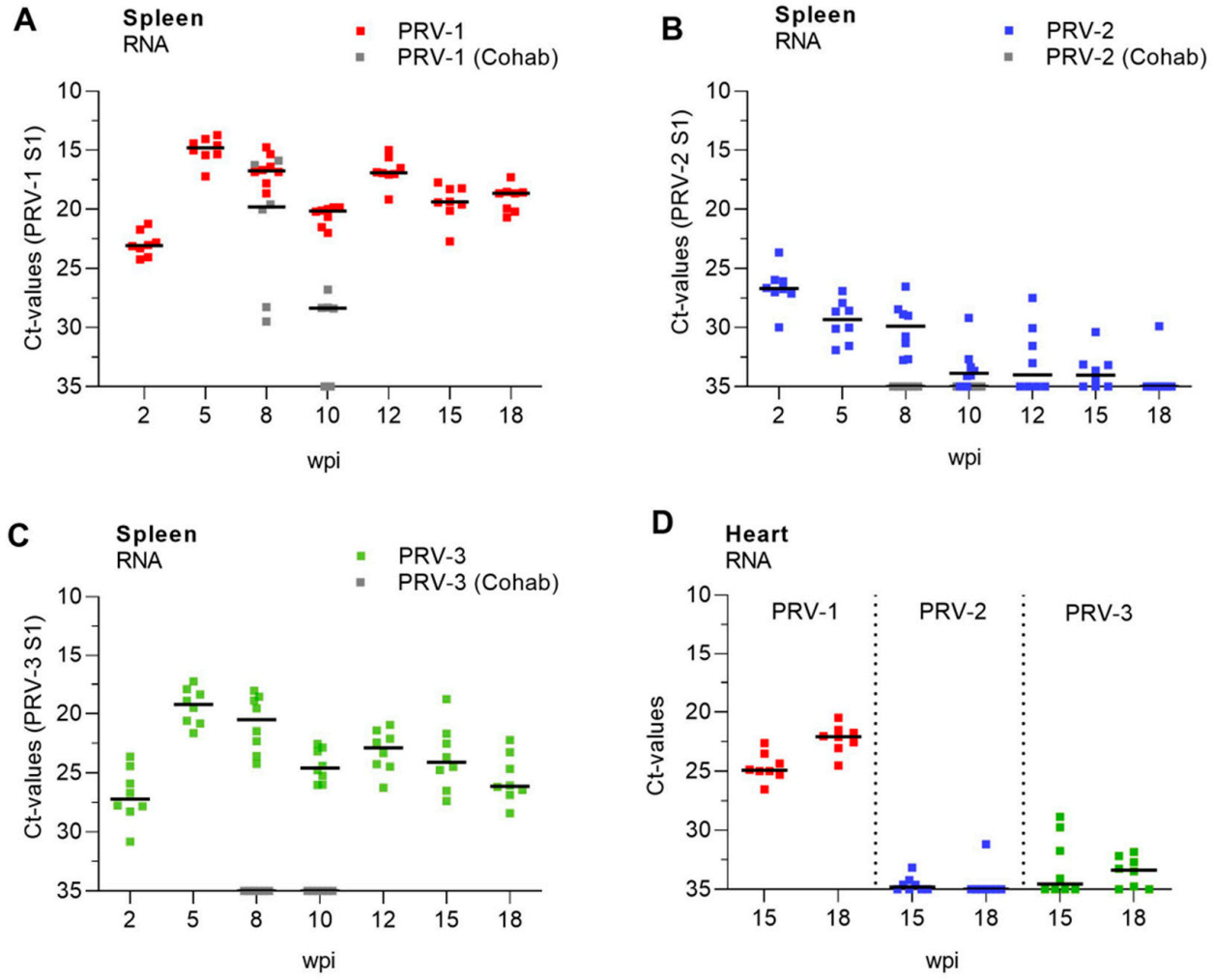


Figure 2. Development of infection with PRV-1, -2 and -3. Levels of PRV-1 (**A**), PRV-2 (**B**) and PRV-3 (**C**) as detected in spleen with specific RT-qPCR assays targeting the S1 genome segment in the respective viruses and trial groups. The figures show individual Ct values and median (line) at each sampling from 2 to 18 weeks post injection (wpi). Gray dots show virus levels in naïve cohabitants added to the tank at 5 wpi and removed at 10 wpi (5 weeks after exposure). Relative levels of PRV-1, -2 and -3 in heart at 15 and 18 weeks post infection (**D**).

To explore if there was any persistence of PRV2 and PRV3 in hearts at the end of the trial, we compared RNA loads of PRV-1, PRV-2 and PRV-3 in heart samples at 15 and 18 weeks (Figure 2D). Whereas PRV-1 levels in heart were below Ct 25, PRV-2 was only detected (median 34.87, IQR Ct 34.31–37.24) in the heart in two fish at 15 weeks after infection, and one fish at 18 weeks. PRV-3 was detected at low levels in 50% of the fish

hearts at both time points. Except for two fish at 15 weeks, all PRV-3-positive fish had Ct levels above 30 in the heart.

3.3. Production of Anti-PRV Antibodies

Using a bead-based multiplexed immunoassay based on recombinant PRV-1 spike protein σ1 and outer capsid protein μ1c [39], the ability of the viruses to induce cross-binding antibodies in plasma (IgM) was explored for the period 2 to 10 weeks after virus injection (Figure 3, Supplementary file S4). PRV-1 infection induced the production of PRV-1-specific antibodies against the viral proteins σ1 and μ1 after 8 and 10 weeks (Figure 3A) and induced unspecific antibodies binding to non-PRV antigens. PRV-2 induced low levels of PRV-1 σ1 binding antibodies as detected at Weeks 5 and 8, declining at Week 10 (Figure 3B), in line with a low PRV-2 replication in the fish. PRV-3 infection induced intermediate levels of PRV-1 σ1 binding antibodies, with lower background binding to non-PRV antigens (Figure 3C). Inactivated PRV-1 did not induce detectable production of antibodies binding to PRV-1 σ1 (Figure 3D).

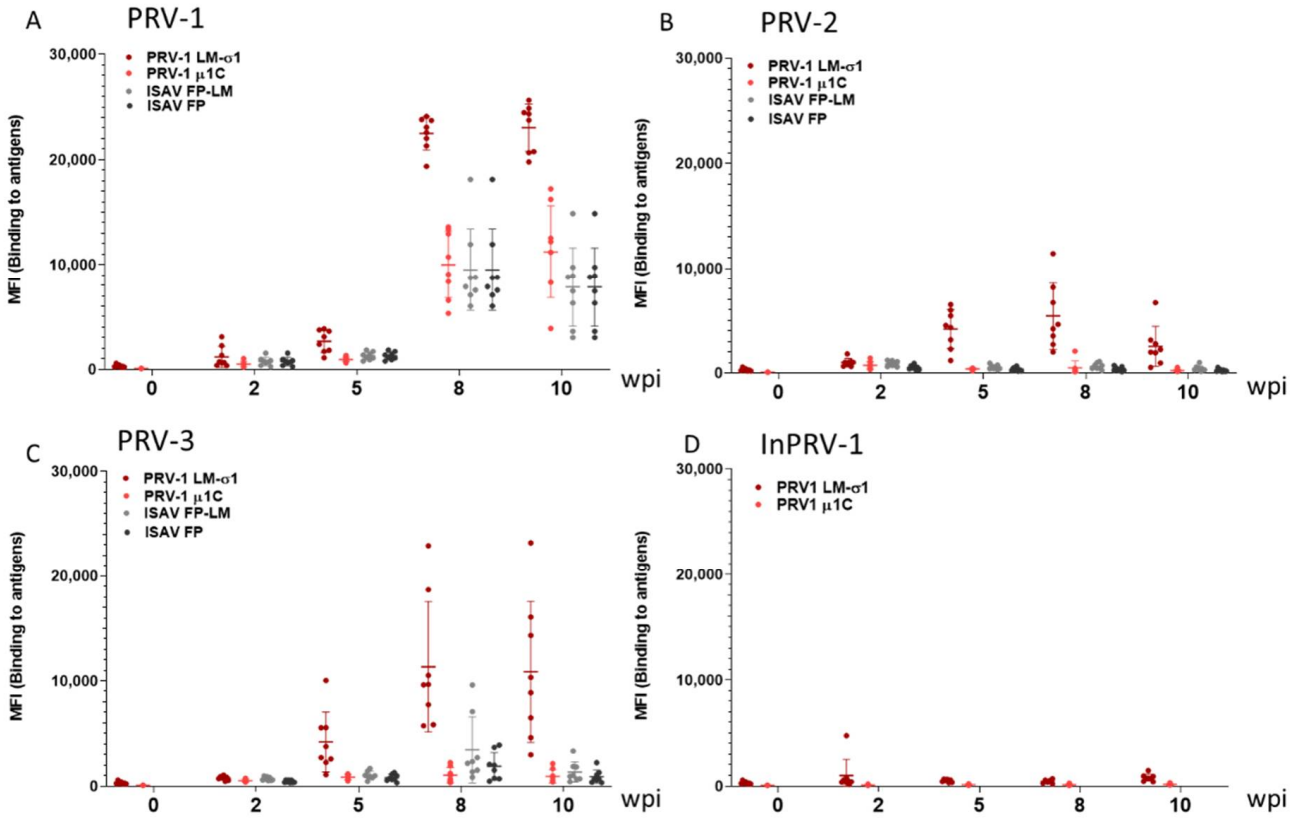


Figure 3. Production of anti-PRV antibodies. Magnetic beads coated with recombinant lipid-modified (LM)-PRV-1-σ1, PRV-1 μ1c, infectious salmon anemia virus fusion protein (ISAV-FP) or LM-ISAV-FP in a multiplexed assay were used to measure PRV-specific and unspecific antibodies in blood plasma sampled from fish in the PRV-1 (**A**), PRV-2 (**B**), PRV-3 (**C**) and InactPRV-1 (**D**) injected groups in the first 10 weeks post injection (wpi). MFI: median fluorescence intensity. The results from beads coated with PRV antigens are shown in red, and beads with non-PRV antigens in gray/black.

3.4. Innate and Cellular Immune Responses

In order to explore which immune responses were activated in the fish at the time of exposure to PRV-1 shedder fish (10wpi), spleen RNA samples were analyzed for transcript markers of cellular cytotoxic immunity (Figure 4, Supplementary file S5): CD8α, IFN-γ and Granzyme A (Figure 4A), and innate interferon-mediated antiviral responses: viperin, myxovirus resistance gene (Mx), and interferon-stimulated gene (ISG)15 (Figure 4B). These genes have previously been shown to be induced in spleen after infection with PRV-1 [37].

PRV-1 infection induced both cellular and innate immune responses in spleen, whereas infection with PRV-2, PRV-3 or inactivated adjuvanted PRV-1 showed no or minor induction of the cellular and selected innate antiviral response genes.

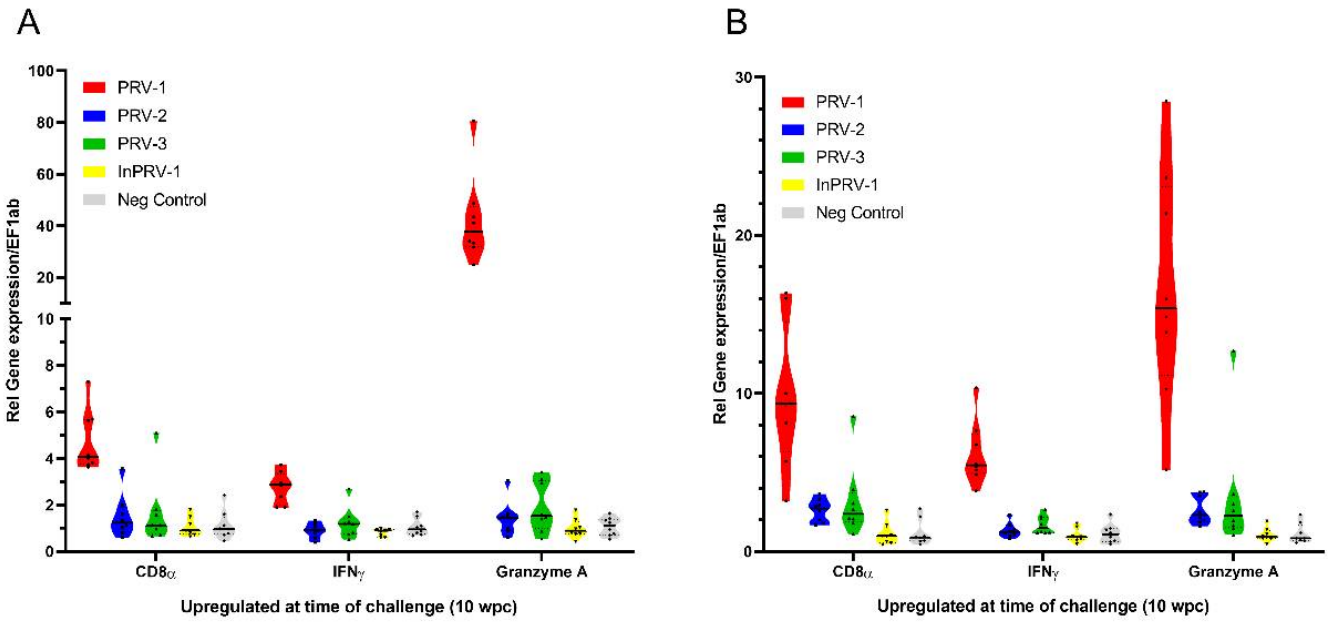


Figure 4. Cellular and innate antiviral immune responses 10 weeks after immunization (wpi). Cellular responses CD8α, IFNγ and Granzyme A (**A**) and innate antiviral responses viperin, myxovirus resistance gene (Mx) and interferon-stimulated gene (ISG)15 (**B**) were analyzed by RT-qPCR in spleen, normalized for the reference gene EF1α and shown as $2^{-\Delta\Delta Ct}$ levels. Gene expression in spleen samples from fish injected with PRV-1 (red), PRV-2 (blue), PRV-3 (green), inactivated PRV (yellow) and mock lysate (gray) are shown.

3.5. Protection from PRV Infection and HSMI

Infection with PRV-1 was monitored in all groups from 12 to 18 wpi (Figure 5A,B, Supplementary file S2). The mock-injected + PRV-1-exposed group acted as a positive control and was infected with PRV-1 after two weeks, peaking 5 weeks later (Experimental Week 15) at median Ct levels of 15 in the spleen and median Ct levels of 17 in the heart. Fish that had been immunized with PRV-2 showed a delayed and variable PRV-1 infection level at 15 and 18 weeks ranging from Ct 15 to 30 in the heart and Ct 10 to 24 in the spleen. Surprisingly, the highest PRV-1 infection levels in the PRV-2 group ranged beyond the levels in the positive controls, indicating that PRV-2 increased susceptibility to PRV-1 infection in some individuals. A similar partial protection was seen in the fish immunized with inactivated, adjuvanted PRV-1 (InactPRV-1), but without the replication boost seen in some fish in the PRV-2 group. In fish infected with PRV-3, the PRV-1 infection was completely blocked, except for two individuals showing high Ct levels in the spleen, one of which also had detectable PRV-3 in the heart.

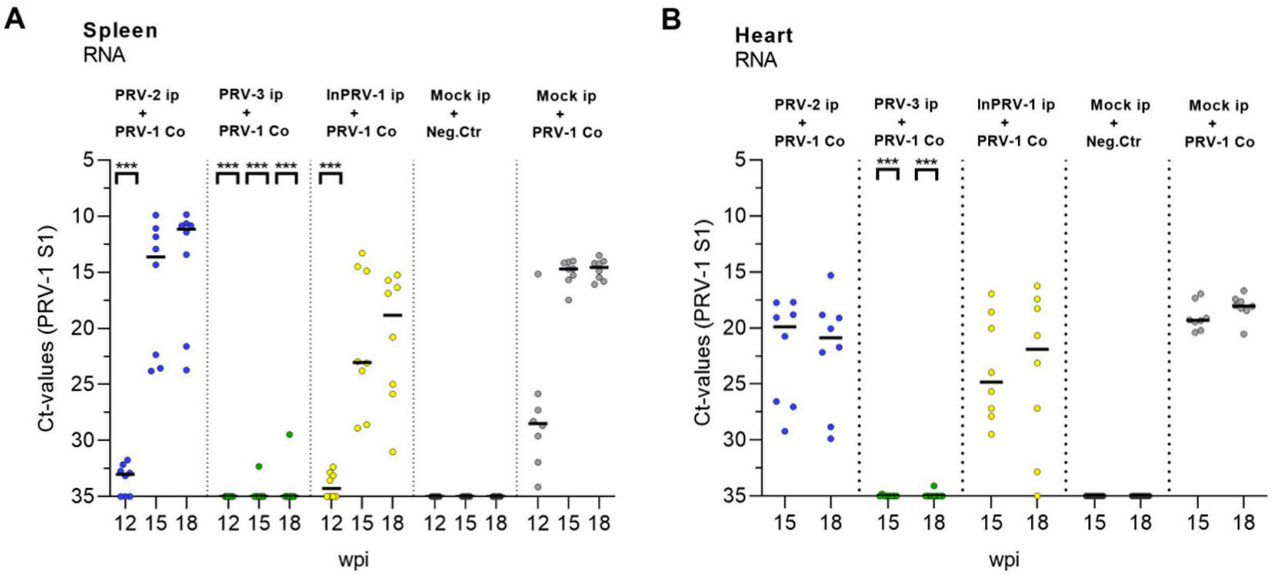
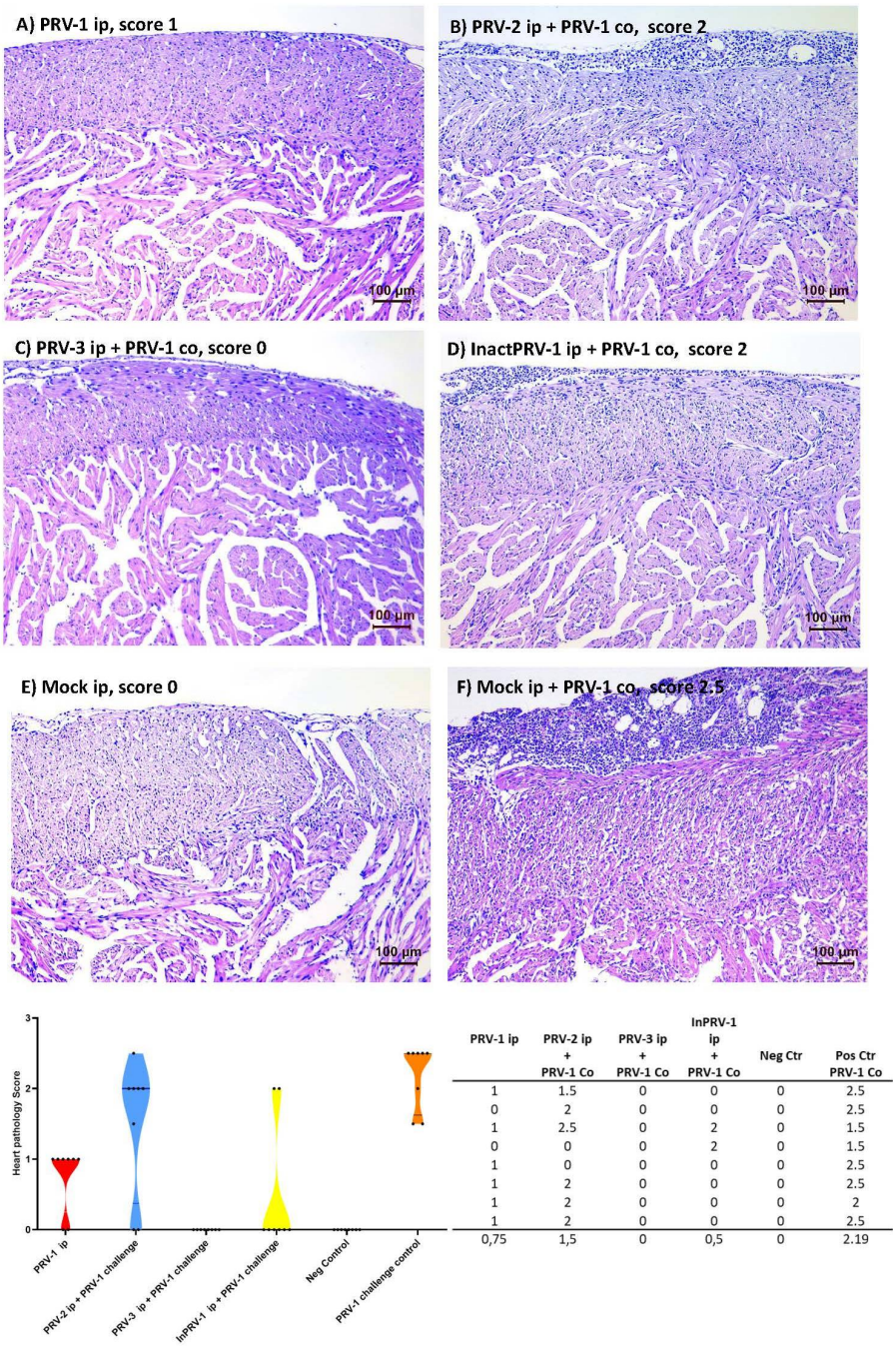


Figure 5. Development of PRV-1-infection after exposure by cohabitation. The level of PRV-1 after infection with PRV-1 shedders at 10 weeks was monitored by RT-qPCR at Weeks 12, 15 and 18 in the spleen (**A**) and Weeks 15 and 18 in the heart (**B**). Each dot represents an individual Ct value with a line (median) at each sampling. Dot color: Fish pre-injected with PRV-2 (Blue), PRV-3 (green), Inactivated PRV-1 (Yellow), or mock (grey), then secondary infected with PRV-1 where marked. Statistical analyses were performed by comparing each target group with the PRV-1 control group at each time point using the Mann–Whitney test. Asterisk shows significant difference (*** $p < 0.001$); wpi = weeks post immunization.

Hearts from fish sampled at 15 and 18 weeks after PRV-1 infection by shedders, and the corresponding uninfected control group, were prepared for histopathology and scored for tissue changes consistent with HSMI (score system 0–3 [11], Figure 6, Supplementary file S6). At 15 weeks, heart pathology was seen only in the PRV-1 group infected ip at the beginning of the trial (five of eight fish had mild lesions, i.e., a score of 1). HSMI-like lesions were present in all individuals in the mock + PRV-1 control group (positive control) at Week 18, with a median HSMI pathology score of 2.5 (1.5–2.5). For the PRV-2 + PRV-1 group, the median pathology score was reduced to 2 (six out of eight fish had lesions), and for the PRV-3 + PRV-1 group pathology was completely absent in all eight fish (a score of 0). Six out of eight fish from the InactPRV-1 + PRV-1 group were also without heart lesions. The group infected with PRV-1 ip Week 0 showed a median pathology score of 1 (six out of eight fish had mild lesions), 18 weeks after infection.



PRV-1 ip	PRV-2 ip + PRV-1 Co	PRV-3 ip + PRV-1 Co	InPRV-1 ip + PRV-1 Co	Neg Ctr	Pos Ctr PRV-1 Co
1	1.5	0	0	0	2.5
0	2	0	0	0	2.5
1	2.5	0	2	0	1.5
0	0	0	2	0	1.5
1	0	0	0	0	2.5
1	2	0	0	0	2.5
1	2	0	0	0	2
1	2	0	0	0	2.5
0,75	1,5	0	0,5	0	2.19

Figure 6. Histopathology and scores of A. salmon hearts. The status of Atlantic salmon hearts was scored for heart and skeletal muscle inflammation (HSMI) pathology 18 weeks after immunization and eight weeks after PRV-1 cohabitant challenge. The scoring of pancarditis was performed according to a visual analogue scale from 0 to 3, where 0 represents a healthy heart, scores above 1 represent hearts with increased cellularity due to immune cell recruitment in the outer epicardial layer, and more severe cases (a score of 2,5) also show increased cellularity in the outer compact and inner spongious layers of the heart ventricle. (**A**) PRV-1 ip injected Week 0, (**B**) PRV-2 immunized ip + PRV-1, (**C**) PRV-3 immunized ip + PRV-1, (**D**) inactivated InPRV-1 immunized ip + PRV-1, (**E**) negative control, mock-injected ip, (**F**) positive control, mock-injected ip + PRV-1, and (**G**) a table and violin plot showing pathology scores of individual fish in each experimental group (n = 8) pre-injected with PRV-1 (red), PRV-2 (Blue), PRV-3 (green), Inactivated PRV-1 (Yellow), or mock (grey), then secondary infected with PRV-1 where marked.

3.6. Immune Responses after Challenge of Immunized Salmon

The specific antibody response (Figure 7A, Supplementary file S4) and cellular cytotoxic immune gene activation—Granzyme A, IFNγ (Figure 7B,C, Supplementary file S5)—were monitored after the PRV-1 challenge at Experimental Weeks 12–18 (two to eight weeks after exposure to shedder fish). The positive control group showed specific and unspecific antibody production and induction of Granzyme A and IFNγ levels in the spleen. The PRV-1-induced antibody response tended to be higher in some fish in the PRV-2 immunized group and lower in fish immunized with inactivated PRV-1. Both observations were in line with the PRV-1 levels found in the spleen. Both groups induced Granzyme A and IFNγ transcripts in fish with high PRV-1 loads, but not in individuals with low PRV-1 loads. In the fish immunized with PRV-3, the antibody levels declined from Week 10 to 18, and since the fish were protected against PRV-1 infection, the antibodies observed most likely resulted from the initial immunization with PRV-3. No regulation of cytotoxic T-cell-associated immune genes was seen. The antibody levels in this group can be compared to the group infected with PRV-1 at Week 0, which showed even higher levels of anti PRV-1 σ1 antibodies during Weeks 12–18. In contrast, whereas fish that were PRV-1 infected Week 0 still had induced levels of Granzyme A in their spleens, the PRV-3-injected group did not.

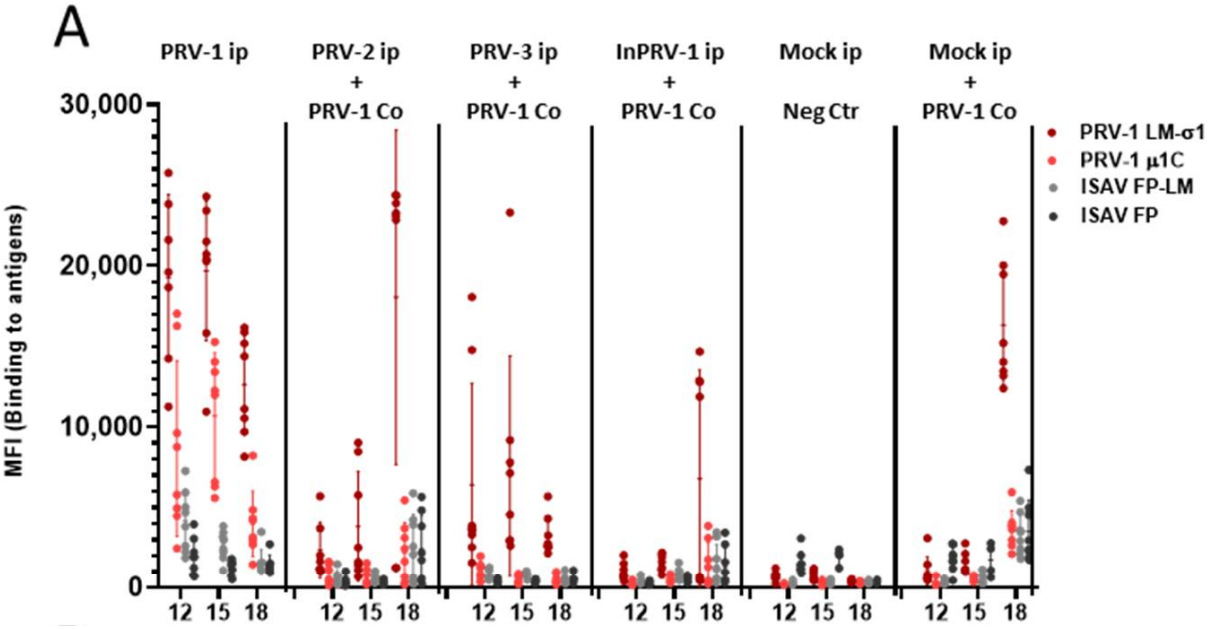


Figure 7. *Cont.*

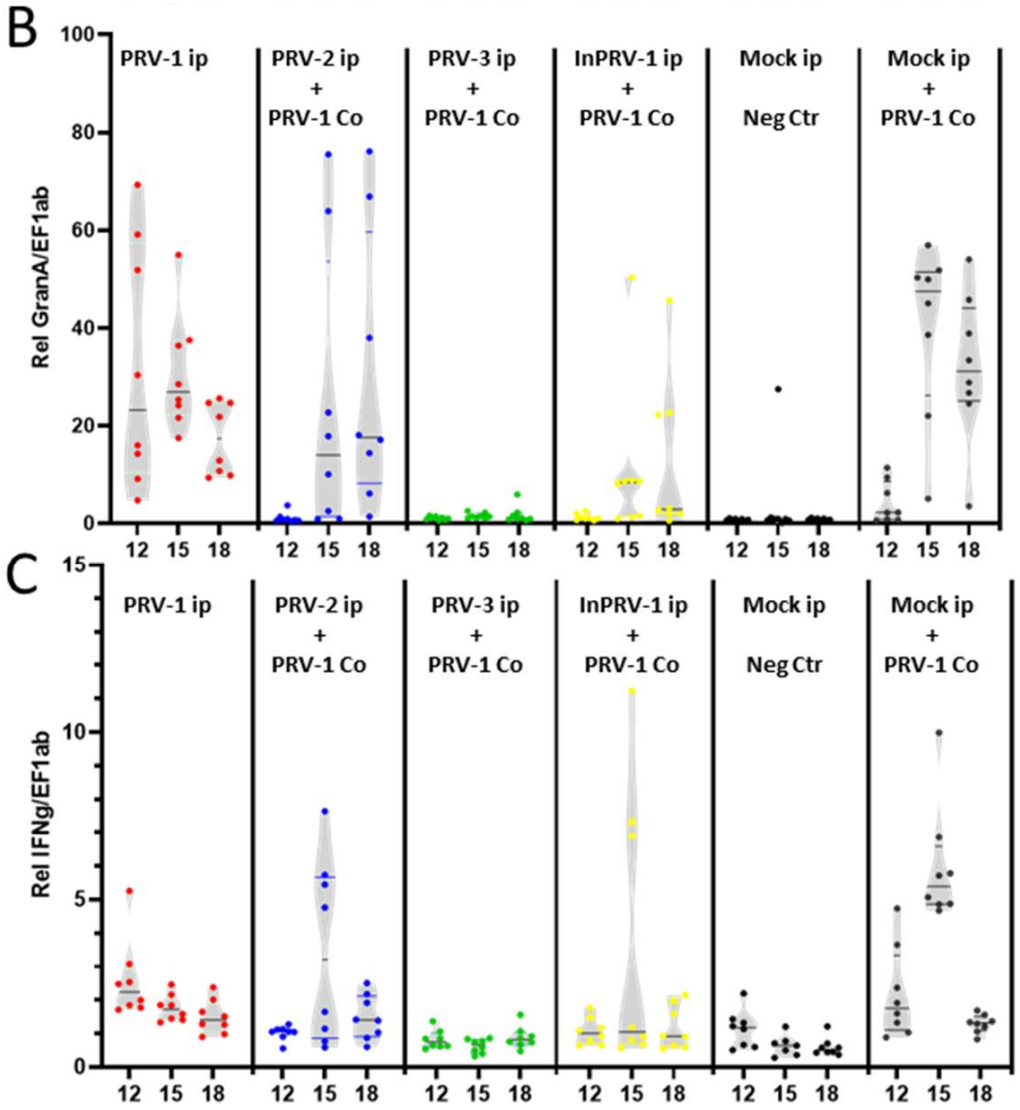


Figure 7. Immune responses in the spleen after PRV-1 challenge of immunized fish. (**A**) Magnetic beads coated with recombinant lipid-modified PRV antigens (LM-PRV-1σ1, PRV-1 μ1c), and non-PRV antigens (ISAV-FP or LM-ISAV-FP), used in a multiplexed assay to measure antibodies from diluted blood plasma sampled from fish in the trial groups. Levels of fluorescent secondary antibody bound to the beads (median fluorescence units, MFI) carrying PRV-antigens (red) and non-PRV antigens (gray/black) were assayed. (**B**) Gene expression of Granzyme A. (**C**) Gene expression of IFNγ in spleen samples from fish injected with PRV-1 (red), PRV-2 + PRV-1 (blue), PRV-3 + PRV-1 (green), InactPRV-1 + PRV-1 (yellow), mock negative control, and mock + PRV-1 positive control groups (black).

4. Discussion

We clarified the potential of the PRV genotypes PRV-2 and PRV-3 to cross-protect against PRV-1 and HSMI, compared them with an inactivated PRV vaccine, and studied some of the possible protective mechanisms involved. Cross-protection induced by related low virulent virus variants was the first successful immunization strategy more than 200 years ago. It was then found that smallpox was prevented by previous exposure to a low virulent pox virus infecting cows [43]. This strategy was used for several years before it was published by Jenner in 1796. A cross-protective approach to immunization introduces many uncertain factors. The theoretical ability of the low virulent virus to cause low-grade disease, develop into virulence over time, or cause disease in other species requires initial mapping and testing. Nevertheless, a replicating mimic of the disease-causing virus itself has the potential of being the ultimate inducer of efficient immune protection, as this will

set off the exact mechanisms used to fight the virus. The rationale for this study is to increase our understanding of cross-protective mechanisms, aiming for the design of more efficient future vaccination approaches.

Although the three PRV genotypes mainly cause disease in different salmonid species, evidence of cross-species infection exists. PRV-1 infect coho salmon, Chinook salmon, and rainbow trout in addition to Atlantic salmon [25], and PRV-3 infects rainbow trout and coho salmon in addition to brown trout [27]. Our observations of experimental infection of Atlantic salmon with PRV-3 confirmed those of a previous study where it was observed that PRV-3 replicated and persisted over a period of 16 weeks and transmitted less efficiently to naïve cohabitants, compared to PRV-1 [29]. Infection with PRV-2, however, is less studied in other species than farmed coho salmon. Here, we show that PRV-2 can infect and replicate in Atlantic salmon after injection, although not as efficiently as PRV-1 and PRV-3. This ability of both PRV-2 and PRV-3 to infect and replicate in Atlantic salmon calls for awareness of all three viruses in aquaculture and breeding.

As previously shown in several previous experimental challenge studies [5,15,44], the PRV-1 genetic variant used in this trial, originating from a Norwegian disease outbreak, induces HSMI in Atlantic salmon. The same ability to cause HSMI experimentally has not been found for Canadian PRV-1 genetic variants [22,23]. The differences in pathogenicity induced by PRV-1 variants was demonstrated experimentally in 2020 [15], and shown to be associated with genetic differences within four out of the ten genetic segments of PRV. Properties of the outer capsid and virus dissemination in the host was suggested as determinants of pathogenicity [15]. Considering the overall similarities between the PRV genotypes at the amino acid level, PRV-1 is more similar to PRV-3 (90% identity) than to PRV-2 (80%) [31]. The most prominent genetic differences were found in the segment S1, encoding the outer clamp protein σ3 and the non-structural protein p13, encoded by an internal open reading frame. These proteins have both been suggested to be implicated in the pathogenicity of PRV [6,45,46], σ3 for promoting virus replication by dsRNA binding and inhibition of the dsRNA-activated protein kinase PKR [47] and p13 for inducing cytotoxicity [45]. The σ3 and p13 proteins are among the least conserved between the PRV genotypes. For PRV2, σ3 and p13 aa identities to PRV-1 homologues are 69.7 and 62.9%, and for PRV-3 the identities are 79.1 and 78.2%, respectively [31]. The rather low aa conservation could potentially be of importance for the host-specific pathogenicity differences of these viruses, or their ability to interact with each other during infection.

When focusing specifically on the amino acid sequence of the outer capsid protein σ1 (S4) from PRV-1, used as antigen in the bead-based immunoassay [39], the identity is 82% with PRV-3 and only 67% with PRV-2 (NCBI database). Since σ1 is considered to be the receptor-binding protein of PRV [6], its sequence variation may explain the species specificity, and the lack of transmission to naïve cohabitants in Atlantic salmon. The higher amino acid identity between PRV-1 and PRV-3, which is in line with their main host species being more closely related, consequently gave a more efficient infection and replication of PRV-3 compared to PRV-2 in Atlantic salmon. A higher rate of virus replication and higher amino acid identity for σ1 as well as for other virus proteins could explain the higher level of cross-binding antibodies detected after PRV-3 infection and thus the higher cross-protecting effect. Although the genetic diversity in PRV-1 is generally not associated with the σ1 gene, it is possible that cross-protection could be different against the genotypes.

In this trial, histological analyses were performed only in the late phase of the trial, i.e., after 18 weeks. At that time, PRV-2 was eradicated from the heart, and PRV-3 levels were low, with Ct values above 30 in 50% of the fish and the remaining fish virus being negative. Compared to this, 100% of the fish injected with PRV-1 at the start of the trial were still virus positive in the heart after 18 weeks, with Ct-levels around 20. We cannot completely rule out that heart inflammation occurred at some point after injection with PRV-2 and PRV-3. In a former study on PRV-3 in Atlantic salmon, minor inflammatory foci were detected in the PRV-3-infected hearts [29], but these findings were not comparable, neither to the inflammation induced by PRV-3 in rainbow trout hearts nor to HSMI in

Atlantic salmon. Infection and pathological changes in other organs, such as the liver and kidney, earlier shown to be sites for PRV replication [21,48,49], or pathological changes at earlier time points in heart cannot be ruled out either, as this was not explored here.

Based on analyses of spleen and heart, PRV-2 appears to be eradicated a few weeks after infection in Atlantic salmon compared to PRV-1 and PRV-3. PRV-2 loads in spleen were similar to those of PRV-3 two weeks after infection, but after 5 weeks PRV-2 levels declined, whereas PRV-3 and PRV-1 levels increased. PRV-3 is reported to be successfully cleared in rainbow trout after infection, not moving into persistence like PRV-1 in Atlantic salmon [29,30]. However, PRV-3 appeared to persist for at least 18 weeks in Atlantic salmon in our study, and also for 16 weeks in a former study [29]. This may indicate that persistence is related to host factors in farmed Atlantic salmon.

In the magnetic bead-based assay used to detect anti-PRV antibodies, the PRV-1 LM-σ1 antigen has earlier been found to be the most efficient antigen for antibody detection [39]. PRV-3 triggered the production of antibodies that were able to cross-bind to PRV-1 LM-σ1. PRV-1 infection has previously been reported to also trigger the production of polyreactive antibodies that bind to non-PRV control antigens [39]. Similarly, we observed high levels of background binding to the ISAV-F-protein control antigens after PRV-1 infection. The production of polyreactive antibodies start at the same time as the more specific antibodies but decrease earlier. The polyreactive antibody response was not seen after PRV-2 or PRV-3 infection. This could be linked to the much higher innate antiviral response triggered by PRV-1, which correlated with the replication efficiency or load of virus for this genotype, compared to the other genotypes. This phenomenon will be subject to further study.

Low levels of antibodies binding to PRV-1 σ1 was observed in blood from PRV-2-injected individuals as well, but only in a short time frame while the virus was still present. Although this low antibody level did not lead to protection from PRV-1 and HSMI, the specificity against PRV antigens and association with virus eradication is notable.

The inactivated, adjuvanted PRV-1 vaccine did not induce any measurable antibodies against PRV-1 σ1. Still, the inactivated PRV-1 vaccine lowered PRV-1 infection levels after secondary challenge, and protected six out of eight individuals from HSMI, in line with previous findings [33]. The mechanism behind this effect is not clear, as neither innate immune activation nor cellular immune activation was revealed through the analyses performed here. We cannot rule out if an early immune activation was triggered by the adjuvant or if antibody-based protection targeting PRV-1 antigens other than PRV-1 σ1 is involved [6]. It is also possible that the inactivation procedure may have changed the structure of the σ1 protein in the inactivated viral particle, as it is in an exposed position in the outer capsid.

The PRV-3 pre-exposure totally blocked PRV-1 infection. Cross-protective antibodies are likely to be one explanation but are most likely not the only one. Several fish had very low levels of detectable antibodies in plasma after 10 weeks, but PRV-1 infection was still completely blocked in these fish. The analysis of expressed antiviral immunity genes and indicators of cellular adaptive immunity (cytotoxic cell markers) did not indicate that these mechanisms were triggered by PRV-3 beyond 10 weeks, at least not in spleen, which was tested. The almost total infection block may lead us to think that protective mechanisms have been induced also at mucosal surfaces, although PRV-3 was given ip and not as a bath exposure. In general, orthoreoviruses enter through respiratory and gastrointestinal mucosal surfaces. Although PRV-1 is reported to infect via the intestinal wall [13], it may use other ports of entry as well. The infection route of PRV-3 has not been studied but could be assumed similar to that of PRV-1. This could point to a mucosal protection mechanism involved in the blockage of PRV-1 infection by PRV-3, which would be a highly desired effect of a future vaccine. Such a PRV-1-blocking effect was not obtained with previous PRV-2 exposure or the inactivated PRV-1 vaccine. It should be noted that PRV-3, but not PRV-2, persisted in the spleen of all fish when they were exposed to PRV-1, and further until the end of the study (18 weeks). It may be that the almost full protection and blocking of PRV-1 infection is dependent on the presence of PRV-3.

All PRV isoforms infect red blood cells, and PRV-1 is shown to strongly induce interferon-regulated antiviral genes in these cells [50]. Thus, the blocking of secondary PRV-1 infection could be a result of red blood cells in an antiviral state. This would be reflected in analysis of spleen antiviral responses. However, very little innate antiviral immune response was induced by PRV-3 in Atlantic salmon although fish were still infected with the virus after 10 weeks. This is remarkably different to a PRV-1 infection, which induces long-lasting antiviral responses. PRV-3 is also reported earlier to induce antiviral responses in rainbow trout [29,30], but not in Atlantic salmon [29]. This difference could be linked to the observed differences in pathogenicity in the two species, but this is still not confirmed and will be further explored.

For PRV-2, 50–80% of the fish had cleared the virus between 10 and 18 weeks after infection. In this group, we found a contradictory effect on PRV-1 infection and HSMI. As two out of eight fish did not develop HSMI, there was no effect on the remaining six. In addition, PRV-1 levels were lower in some fish, but strongly boosted in others. It appeared that PRV-1 may have replicated more efficiently in some of the individuals that had eradicated PRV-2, compared to individuals that had not. Like for PRV-3, we did not detect innate antiviral immune responses to PRV-2 infection 10 weeks after infection.

PRV-1 induces cytotoxic T-cell (CTL) activity in Atlantic salmon [17,37], which is strongly associated with HSMI pathology [16], and also heart inflammation in rainbow trout infected with PRV-3 [30]. Here, there is clear evidence that PRV-1 induces a strong regulation of innate antiviral and cytotoxic immune response genes 10 weeks after infection, which is not induced by PRV-2 or PRV-3, and which is likely to be decisive for HSMI pathology. The role of CTL activity in vaccine effects and long-term protection against viruses in salmonids is not much studied, but specific cytotoxicity against the salmonid alphavirus (SAV) has recently been explored after vaccination with an adjuvanted inactivated SAV vaccine, in comparison with SAV infection [51]. There, it was clearly demonstrated that while SAV infection induced specific cytotoxicity, only unspecific cytotoxic activity was induced by the vaccine [51]. It would, in a follow-up study, be interesting to compare specific CTL activity in the period after PRV-2 and PRV-3 infection to explore any correlation with the ability to cross-protect against PRV-1.

This study illustrates some potential pitfalls in using replicating viruses for vaccine purposes. They may be very efficient, like PRV-3, which completely blocks PRV-1 infection. However, PRV-3 itself persists in the fish, which may have unknown long-term consequences.

This study also indicates that antibodies against the putative receptor-binding protein σ1 may be an important protective measure. PRV-3, but not PRV-2, induced the production of anti-σ1 antibodies. This could be due to the higher replication rate of PRV-3 to PRV-2 in Atlantic salmon and the higher identity between the σ1 protein of PRV-1 and -3. A protective effect could eventually be verified in a passive immunization test by administration of purified serum immunoglobulin from PRV-3-infected fish to PRV-1 experimentally infected fish. The long-term protective effects of these antibodies will be subject to follow-up experiments, as we could observe a decline after > 15 weeks of PRV-3 infection. If plasma antibodies are also involved in blocking infection at mucosal surfaces is an open question.

PRV-2 replicates at low levels in Atlantic salmon and is eventually cleared, which normally could be considered beneficial properties of a "live" replicating vaccine. However, the replication must be at a level sufficient to induce an effective immune response. Here, only minor innate and cellular responses were found at the transcript level. However, there may be effects at the post-transcriptional or post-translational levels that we did not monitor. PRV-2 caused contradictory results by protecting some fish from HSMI but causing even higher susceptibility to PRV-1 infection in others. The large individual differences could possibly be due to host genetics, leading to a different ability to present antigen. This study also confirms the partial efficiency of the inactivated PRV-1 vaccine published earlier; although, it is still without a clear answer to the main mechanism of protection.

Besides the obvious pitfalls in immunizing Atlantic salmon against HSMI with PRV-3, a virus pathogenic to rainbow trout [28], there are also additional concerns associated with a live attenuated vaccination approach. Segmented RNA viruses may reassort or recombine if two related genotypes infect the same cell [52], creating new viruses with unpredictable properties, potentially pathogenic.

Future vaccine production can provide us with reverse genetic approaches and viruses tailored by synthesis and gene editing. Combined with thorough long-term studies of risks and effects of the different vaccine approaches and a higher repertoire of ways to measure vaccine effect, this will hopefully ensure safe and effective attenuated vaccines in the future.

5. Conclusions

This work show that PRV-1, PRV-2 and PRV-3 replicate in Atlantic salmon, and can induce production of antibodies that bind to the PRV-1 σ1 antigen. Only PRV-1 in-fection induce unspecific antibodies, long-lasting expression of antiviral response genes and cytotoxic genes in spleen in Atlantic salmon, which could be associated with the ability to cause HSMI. When compared to vaccination with an inactivated PRV-1 vaccine, PRV-3 infection provides full protection from PRV-1 introduced ten weeks later, and development of HSMI. In comparison, inactivated PRV-1 vaccine and PRV-2 infection does not prevent PRV-1 infection and only partially protects against HSMI. This work indicates that a replicating attenuated vaccine approach could protect against HSMI.

Supplementary Materials: The data are available online at https://www.mdpi.com/2076-393X/9/3/230/s1, Table S1: Primer and probe sequences (5′-3′) for PRV genotypes and immune genes, Supplementary File S2: PRV Ct values and weight data from the PRV immunization trial, Figure S1: Infection level of PRV genetic variants in all groups 2, 5 and 10 weeks post injection (wpi), control of cross-infection. Supplementary File S4: Antibody binding to PRV antigens given as mean fluorescence intensity (MFI) corrected for binding to control beads, Supplementary File S5: Immune gene expression data, Supplementary File S6: Histopathology scores of heart samples.

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Data Availability Statement: The supplementary data files presented in this study are openly available in Zenodo, at https://doi.org/10.5281/zenodo.4450287 (accessed on 6 March 2021).

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