Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Food Safety and Infection Biology

Philosophiae Doctor (PhD)
Thesis 2020:01

Detection of antibodies in Atlantic salmon using a bead-based multiplexed immunoassay

Deteksjon av antistoffer i atlantisk laks med et kulebasert multipleks immunoassay

Lena Hammerlund Teige

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“I know of no pleasure deeper than that which comes from contemplating the natural world and trying to understand it.”

— Sir David Attenborough

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Oslo, November 2019
Lena Hammerlund Teige

I must also acknowledge what first sparked my interest in immunology at the age of seven: the cartoon "Once Upon a Time...Life" and especially the brave B cell, ***Lieutenant Psi****.*
(Image modified from unknown sources)

ABBREVIATIONS

A	
AID	Activation-induced cytidine deaminase
AMP	Antimicrobial peptide
APC	Antigen presenting cell
B	
BAFF	B cell-activating factor
BCR	B cell receptor
C	
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CDR	Complementarity-determining region
CHSE	Chinook salmon embryo (cells)
CTL	Cytotoxic T lymphocytes
D	
DNP	Dinitrophenol
dPBS	Dulbecco's phosphate-buffered saline
dsRNA	Double-stranded RNA
E	
Ef1	Elongation factor 1α
EIBS	Erythrocytic inclusion body syndrome
Eif3ea	Eukaryotic translation initiation factor 3 subunit E-A
ELISA	Enzyme-linked immunosorbent assay
F	
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FDC	Follicular dendritic cell
H	
HSMI	Heart and skeletal muscle inflammation
I	
i.p.	Intraperitoneal
IFN	Interferon
IHN	Infectious hematopoietic necrosis
ILT	Interbranchial lymphoid tissue
IPNV	Infectious pancreas necrosis virus
ISVP	Infectious subviral particle
J	
JAM-A	Junction adhesion molecule-A
K	
KLH	Keyhole limpet hemocyanin
L	
L-15	Leibovitz's L-15 medium
LM	Lipid-modified
LPS	Lipopolysaccharide
M	
MAC	Membrane attack complex
MALT	Mucosa-associated lymphoid tissues
MHC	Major histocompatibility complex
MMC	Melanomacrophage center
MRV	Mammalian orthoreovirus
N	
NK cell	Natural killer cell
NLR	NOD-like receptor
O	
ORF	Open reading frame
P	
PAMPs	Pathogen-associated molecular patterns
PD	Pancreas disease
PE	Phycoerythrin
PIgR	Polymeric immunoglobulin receptor
PRRs	Pattern recognition receptors
PRV	*Piscine orthoreovirus*
R	
RAG	Recombination-activating gene
rsp20	Ribosomal protein 20
RT-qPCR	Reverse transcription quantitative/real-time polymerase chain reaction
S	
SALT	Skin-associated lymphoid tissue
SAV	Salmonid alphavirus
SD	Sleeping disease
T	
Tat	Twin arginine translocase
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TLRs	Toll-like receptors
TNP	Trinitrophenol
V	
VHSV	Viral hemorrhagic septicemia virus
W	
wpc	Weeks post challenge

LIST OF PAPERS

Paper I

A bead based multiplex immunoassay detects *Piscine orthoreovirus* specific antibodies in Atlantic salmon (*Salmo salar*)

Authors: Lena Hammerlund Teige, Morten Lund, Hanne M. Haatveit, Magnus Vikan Røsæg, Øystein Wessel, Maria K. Dahle, Anne K. Storset

Published: Fish & shellfish immunology 63 (2017) 491-499

Paper II

Detection of salmonid IgM specific to the *Piscine orthoreovirus* outer capsid spike protein sigma 1 using lipid-modified antigens in a bead-based antibody detection assay

Authors: Lena Hammerlund Teige, Subramani Kumar, Grethe M. Johansen, Øystein Wessel, Niccolò Vendramin, Morten Lund, Espen Rimstad, Preben Boysen, Maria K. Dahle

Published: Frontiers in Immunology 10 (2019) 2119

Paper III

IgM directed against Salmonid alphavirus antigens can be detected after a natural pancreas disease outbreak, using a bead-based immunoassay

Authors: Lena Hammerlund Teige*, Ida Aksnes*, Magnus Vikan Røsæg, Ingvill Jensen, Jorunn Jørgensen, Hilde Sindre, Espen Rimstad, Maria K. Dahle, Preben Boysen.

Manuscript

**Equal contributions*

SUMMARY

Viral diseases are among of the main challenges in aquaculture in Norway today. Heart- and skeletal muscle inflammation, caused by Piscine orthoreovirus (PRV), and pancreas disease (PD), caused by Salmonid alphavirus (SAV), both cause huge losses of farmed Atlantic salmon. Vaccination is a possible solution that is already contributing to the control of bacterial diseases. Unfortunately, making effective vaccines against viral diseases has been challenging and information about which immune mechanisms that are involved in protection is lacking.

Antibodies can mediate complete protection against some diseases, and antibody levels after vaccination can correlate with protection, even if the protection is mediated by other mechanisms. In addition, antibody detection can be used in disease surveillance to determine if a fish population is or has been infected with a virus. Unfortunately, measuring antibody levels in salmon is not straightforward. Such measurements have not been widely used, and often show high levels of background binding. An important reason for this is that fish antibodies are of the IgM type. IgM is less specific than IgG, the dominating antibody in mammals. Therefore, other methods, like PCR and histology, are more used in diagnostics in Atlantic salmon. In this work, we have for the first time used an assay based on microscopic magnetic beads conjugated with antigen to measure antibody levels in Atlantic salmon. This method has a high sensitivity and can be used to measure antibodies against several proteins simultaneously in the same sample.

By using this method, we have detected antibodies against PRV and SAV. In paper I and II, plasma from two PRV challenge trials was used to detect antibodies against the PRV proteins µ1c, µNS and σ1. We also detected antibodies against PRV1- σ1 in plasma from PRV-3-infected rainbow trout. There have been no previous publications detecting antibodies against PRV. The peak antibody level coincided with decreased pathology in the heart. In addition to showing virus-specific antibodies, our results show an increase in non-specific antibodies in PRV-infected salmon. This unspecific binding, but not the virus-specific binding, was decreased by heat treatment of samples. The non-specific antibodies could be so-called polyreactive antibodies. Polyreactive antibodies could be crucial in protecting the salmon against infections before the adaptive immune system has had time to react, but their function, as well as the function of virus-specific antibodies during a PRV infection is unclear.

In paper III, we detected antibodies against whole SAV particles disrupted with Triton-X. The antibody binding increased from between week three and week six after the introduction of SAV-injected shedder fish. The SAV particles also worked well with little background binding when analyzing plasma from a PD outbreak in the field. Antibodies were detected in most fish from four

weeks after the start of the outbreak, and the antibody level stayed elevated until the last sampling point at 15 weeks. At this time point, most samples were negative for virus when analyzed with RT-qPCR, showing that serology has a longer window of detection compared to detection of viral RNA by RT-qPCR.

These results show that both virus-specific and non-specific antibodies can be produced after infection in Atlantic salmon. The method used is well suited for antibody detection in salmon but can be complicated by the presence of non-specific antibodies. It is therefore crucial to optimize the antigens for detection of specific antibodies.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

Virussykdommer er en av de store utfordringene i oppdrettsnæringa i Norge i dag. Hjerte- og skjelettmuskelbetennelse, forårsaket av *Piscine orthoreovirus* (PRV), og pankreas sykdom (PD), forårsaket av Salmonid alphavirus (SAV), fører begge til store tap av atlantisk laks i oppdrett. Vaksinering er en mulig løsning som allerede har bidratt til god kontroll på bakterielle infeksjoner hos laks. Dessverre er det utfordrende å lage vaksiner som virker godt mot virusinfeksjoner. Informasjon om hvilke immunfunksjoner som er involvert i beskyttelse er også mangelfull.

Antistoffer kan gi full beskyttelse mot noen sykdommer, og antistoffnivået etter vaksinering kan korrelere med beskyttelsen, også om beskyttelsen skyldes andre immunmekanismer. I tillegg kan antistoffdeteksjon brukes i sykdomsovervåkning for å finne ut om fiskepopulasjoner er eller har vært infisert med et virus. Dessverre er det ikke rett fram å måle antistoffnivå hos laks. Antistoffmåling er lite brukt og det er ofte mye bakgrunnsbinding. En viktig grunn til dette er at fiskens antistoff er av typen IgM, som er mindre spesifikk enn IgG i pattedyr. Derfor er andre metoder, som PCR og histologi, mest brukt til påvisning av virussykdom hos laks. I dette arbeidet har vi for første gang brukt et assay basert på mikroskopiske magnetiske kuler konjugert med antigen til å måle antistoffnivå i laks. Denne metoden har høy sensitivitet og gir muligheten til å måle antistoffer mot mange proteiner i samme prøve samtidig.

Ved å bruke denne metoden har vi detektert antistoffer mot PRV og SAV. I artikkel I og II ble plasma fra to smitteforsøk med PRV brukt for å påvise antistoffer mot PRV-proteinene µ1c, µNS og σ1. Vi har også påvist antistoffer mot PRV1-σ1 i plasma fra regnbueørret infisert med PRV-3. Det er ikke publisert andre tester for deteksjon av PRV-antistoffer tidligere. Nivået av antistoffer var på topp samtidig som graden av patologiske forandringer i hjertet gikk ned. I tillegg til å vise virus-spesifikke antistoffer viser våre funn en økning i uspesifikke antistoffer i PRV-infisert laks. Denne uspesifikke bindingen ble redusert ved varmebehandling av plasma, uten at den spesifikke bindingen til PRV ble redusert. De ikke-spesifikke antistoffene kan være såkalte polyreaktive antistoffer. Polyreaktive antistoffer kan være avgjørende for å beskytte laksen mot infeksjoner i tiden før det adaptive immunsystemet har fått tid til å reagere, men det er usikkert hvilken funksjon både disse og de virusspesifikke antistoffer har ved en PRV-infeksjon.

I artikkel III detekterte vi SAV-spesifikke antistoffer ved bruk av hele SAV-partikler, behandlet med Triton-X, som antigen på kulene. Antistoffbinding til kulene økte fra mellom uke 3 og uke 6 etter introduksjon av SAV-injiserte shedderfisk i et smitteforsøk. Disse antigenene fungerte også godt, med lite bakgrunnsbinding, da plasma fra fisk i et PD-utbrudd i felt ble undersøkt. Antistoffer kunne detekteres i de fleste fisk fra fire uker etter at utbruddet begynte og antistoffnivået holdt seg høyt til

siste prøvetidspunkt ved 15 uker etter utbruddet. På dette tidspunktet var de fleste prøver negative for virus målt med RT-qPCR. Dette viser at vinduet for SAV-diagnostikk er lengre ved bruk av serologi enn ved deteksjon av virus RNA med RT-qPCR.

Disse forsøkene viser at det kan produseres både virus-spesifikke og uspesifikke antistoffer etter infeksjon hos laks. Metoden vi har brukt er godt egnet til antistoffdeteksjon, også i laks, men ikke-spesifikke antistoffer kan komplisere bruken. Det er derfor avgjørende å finne gode antigener som i størst mulig grad detekterer spesifikke antistoffer.

1 INTRODUCTION

1.1 BACKGROUND

Atlantic salmon, *Salmo salar* is the most important fish in Norwegian aquaculture. Norway is the dominant Atlantic salmon producer, with 1,25 million tons slaughtered in 2018 (Norwegian Veterinary Institute, 2019). This equals the weight of 5000 Statue of Libertys, or more than three times the weight of the entire Norwegian population. Other major Atlantic salmon producers are Chile, USA, Canada, Scotland, Iceland, Faroe Islands and Australia. Farmed Atlantic salmon have a small carbon footprint with its low feed-conversion ratio and is therefore a biologically-efficient source of food and animal protein (Torrissen *et al.*, 2011). However, for the industry to be sustainable, it must address issues like viral diseases, fish welfare, sea lice, conservation of wild fish stocks, sustainable feed and minimizing the environmental impact.

Every year around 50 million salmon is lost during the sea water phase (Norwegian Veterinary Institute, 2019). Better disease control is important to reduce this number. Disease is incompatible with good animal welfare and there are huge economical losses associated with disease. A high loss of fish will also increase the environmental footprint of the industry. Heart and skeletal muscle inflammation (HSMI) caused by *Piscine orthoreovirus* (PRV) and pancreas disease (PD) caused by Salmonid alphavirus (SAV) are considered two of the most important viral diseases. Both diseases cause varying mortality, often between no mortality and 20 % for HSMI and from insignificant to over 60 % for PD. In addition to HSMI, PRV is associated with melanized spots in salmon filets, causing economic losses, as these filets are downgraded. PD can cause a necrosis of the exocrine pancreas that reduces feed conversion and significantly impact growth. Both viruses also contribute to increased mortality following stressful events like handling and delousing.

Fighting viral infections in aquaculture is largely based on avoiding contact between the disease producing viruses and the susceptible hosts. Vaccination is an effective way of controlling diseases in species with an adaptive immune system and have been used to control and eradicate human and animal diseases, and to successfully control bacterial diseases in aquaculture. Today viral diseases represent one of the biggest challenges in the global aquaculture industry (Walker & Winton, 2010). There is no treatment available against viral diseases in Atlantic salmon. Vaccination is consequently the only way, in addition to biosafety measures and breeding strategies, to control viral diseases in aquaculture. There are few vaccines against viral diseases in use, and those available give a suboptimal protection (Sommerset *et al.*, 2005, Biering *et al.*, 2005, Gomez-Casado *et al.*, 2011). B cells and antibodies play a key part in native and vaccine induced defense against infections. In order

to make good vaccines, i.e. vaccines that elicit a strong innate response, induce long-lasting immune protection and produce no adverse reactions; a better understanding of both the pathogen, the salmon immune system and the interaction between them is required. There is also a need to find good correlates with protection. Antibody level is suggested to be the most reliable correlate of protection from vaccination (Munang'andu & Evensen, 2019). To understand the host-pathogen interactions and mechanisms involved in protective immunity in salmonids, both the B and T cell responses need further characterization. These mechanisms are incompletely understood in fish, and assumptions are often based on what is known of the mammalian, especially the human and murine immune system. Immunological research in Atlantic salmon has lacked tools, including good monoclonal antibodies, to describe key elements involved in immunological protection.

Serology has not been commonly used for diagnostics in fish, as fish antibodies are often cross-reactive. There has been some use of enzyme-linked immunosorbent assays (ELISA) for antibody detection, but high background titers and low specific responses are often found. Neutralization assays can be used for viruses that can be propagated in cell lines but are not used for routine diagnostics in Norway. A multiplex immunoassay with antigens from several pathogens has been suggested as a possible tool for screening and diagnostic purposes in aquaculture (Adams & Thompson, 2008). Multiplex bead-based immunoassays are more sensitive than ELISAs and could potentially be used alone or together with RT-qPCR in diagnostics, for surveillance purposes, to evaluate vaccine efficacy and to investigate the immune and host-pathogen responses. They have not previously been used for this in Atlantic salmon.

Teleost fish diverged from the mammalian line around 440 million years ago. Evolutionary, fish were the first animal group to possess both an innate and an adaptive immune system. In addition to benefiting the aquaculture industry, studying the immune system of fish is important to understand shared immunological mechanisms in vertebrates and the evolution and diversification of the adaptive immune system. Discoveries made in fish have greatly influenced the field of immunology in the past and will likely continue to do so.

1.2 THE IMMUNE SYSTEM

The immune system is made up of a network of specialized organs, cells and molecules that cooperate to detect invading microorganisms, parasites and toxins, and protect the body against them. A fundamental prerequisite for this is the ability to discriminate between self and non-self, or more correctly between what is dangerous (can be both self and non-self) and what is not (Janeway, 1992, Matzinger, 1994). The immune system can be divided into the innate and the adaptive (also called the acquired or the specific) immune system, and into the cellular and the humoral immune system. In reality these systems are deeply integrated with each other. The innate immune system is the first line defense, and is comprised of anatomical barriers, cells and molecules already in place and ready to respond to a pathogen. The innate immune system responds before the adaptive responses have had time to develop (figure 1). It also helps in the elimination of damaged cells and in tissue repair, and it stimulates and guides the direction of the adaptive immune responses. Some form of innate immune system is present in plants, fungi, invertebrates and primitive multicellular organisms (Zasloff, 2002, Beutler, 2004). In addition to this, vertebrates also possess an adaptive immune system. The humoral and cellular parts of the adaptive immune system are dependent on both the innate immune system and on each other for optimal protection of the host. The adaptive part of the immune system is able to change depending on the pathogen encountered and can also "remember" previously encountered pathogens to better fight them the next time around.

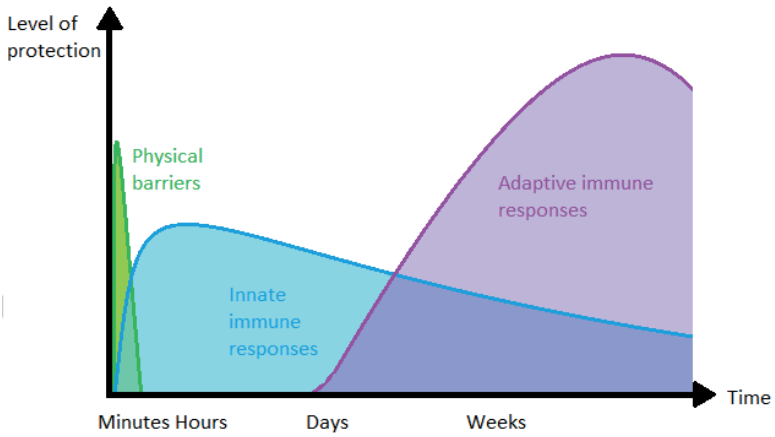

Figure 1. *The time course of protective features against infecting microorganisms. Immediate protection by physical barriers at the body surface, rapid protection by innate mechanisms, and adaptive mechanisms developing over time.*

Innate immunity

For a pathogen to invade and establish an infection it first must pass through physical barriers protecting the host from the environment. Examples of such barriers in mammals are the skin and the mucosal membranes in the respiratory organs and the gastrointestinal tract. These barriers are covered with endothelial cells with tight junctions, physically blocking the entry of microbes between cells. Mucosal surfaces have a layer of viscous mucus and a fast cell turnover, and there can be peristaltic movements helping in the elimination of microbes. In addition to the physical properties of these surfaces, the epithelial cells produce a number of antimicrobial molecules, and a high number of special cells of the innate immune system are present here (Abbas *et al.*, 2015).

The innate immune system recognizes molecules and structures that are essential parts of pathogens, but not found in the host. These structures are called pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are the double-stranded RNA found in viruses, lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria and polysaccharides (like mannoses residues) found in glycoproteins from bacteria and fungi. Most cells possess receptors capable of recognizing PAMPs, called pattern recognition receptors (PRRs), but phagocytic cells possess the widest range of such receptors. Important PRRs are toll-like receptors (TLRs), NOD-like receptors (NLRs) and Retinoic acid-inducible gene (RIG)-like receptors. TLRs are found on cell membranes and endosomal membranes of several cell types and recognizes, among others, LPS, bacterial lipoproteins and viral nucleic acids. NLRs are found in cell cytosol and recognize bacterial peptidoglycans. RIG-like receptors are found in the cytosol and recognize viral RNA. PRRs are linked to intracellular signaling pathways and upon binding to their microbial ligand trigger a signaling pathway leading to the activation of different transcription factors. These transcription factors induce nuclear gene expression followed by production of inflammatory and/or antiviral proteins (Abbas et al., 2015). Phagocytic cells like neutrophils, macrophages and dendritic cells play an essential role in the innate immune system. Their primary function is to engulf and destroy microbes. They also communicate with other cells to regulate the immune response. Macrophages reside in most tissues and can be recruited from blood monocytes that differentiate into macrophages in an inflammatory environment (Yang *et al.*, 2014). Natural killer (NK) cells and other innate lymphoid cells are also part of the innate immune system. NK cells can detect and kill virus infected cells. The cells of the innate immune system communicate with the adaptive immune cells through secreted cytokines and direct contact between cell surface receptors. If a pathogen succeeds in bypassing the mucosal defense mechanisms and enters the blood it will encounter the complement system. The complement system is an important cascade system and consists of over 35 complement and regulatory proteins (Volanakis, 1998). Complement can be activated through three pathways, the

classical pathway, the alternative pathway and the lectin pathway. All pathways generate factor C3. The proteins react against pathogens through a cascade ending with opsonization of the pathogen, enhanced inflammatory responses and the formation of a membrane attack complex (MAC) (Riera Romo *et al.*, 2016).

Adaptive immunity

The fight between pathogens and their hosts is a constant evolutionary arms race between microbes with high rates of mutation and variation, and long-lived hosts with much lower rates of mutations (Haldane, 1949). Compared to bacteria and virus, vertebrates are slow to reproduce and cannot compete with the rapid evolution of viruses and bacteria. A normal generation interval for bacteria can be 20 minutes, leading to frequent mutations. Viruses also have a high rate of mutations, especially RNA viruses, as RNA polymerases lack the proofreading ability of DNA polymerases. Some of these mutations will be beneficial for the pathogen and some will increase its virulence. With the adaptive immune system comes adaptation and increased diversity, making it possible to respond to variations in pathogens within time scales significantly shorter than the host's generation time.

Fundamental features of the adaptive immune system are antigen specificity and the ability to acquire long-lived memory. This part of the immune system is activated upon contact with an antigen. An antigen is nothing else than a foreign molecule – like a PAMP, but more diverse as almost anything can be an antigen. Four key antigen receptors form the basis of the adaptive immune system, all members of the immunoglobulin superfamily, constructed by immunoglobulin domains and binding antigens through the use of variable domains. They are the B cell receptor (BCR), the T cell receptor (TCR), the major histocompatibility complex class I (MHC-I) and the major histocompatibility complex class II (MHC-II) (Tizard, 2013).

The cells of the adaptive immune system are the B and T lymphocytes, also called B cells and T cells. T cells only recognize antigens bound by MHC molecules on the surface of other cells. Peptides derived from phagocytosed or newly synthetized cytosolic protein are bound to MHC molecules before they are displayed together on the cell membrane. All T-cells possess a TCR consisting of two peptide chains. In mammals these can be either an α with a β chain or a γ chain with a δ chain. In mammals, the α/β T-lymphocytes can be divided into cytotoxic T cells and helper T cells (T_h cells) that in addition to the TCR possess co-receptors CD3 and CD8 or CD4, aiding in the binding to MHC. The cytotoxic CD8+ T cells interact with the MHC-I molecules. MHC-I is present on most cells and present short peptide chains, usually 9 amino acid residues long, derived from cytosolic proteins. Therefore, only endogenous proteins and proteins from intracellular pathogens are presented on

MHC-I. An activated cytotoxic T cell kills the cells that present a foreign peptide if the TCR binds to it with sufficient affinity, thereby eliminating intracellular pathogens. The CD4+ T cells can bind with MHC-II molecules. Only cells that can endocytose extracellular molecules or pathogens, like macrophages, dendritic cells and B cells, express MHC-II. These cells are therefore called professional antigen-presenting cells (APC). After endocytosis, proteins are processed and presented on MHC-II as 9-12, or longer amino acid peptides. CD4+ cells (T_h cells) are activated when they bind to an antigen presented on the MHC-II of an APC. When this happens, a signaling cascade is started. Different cytokines can be produced that are able to skew the direction of the immune response. The activated effector T_h cell can activate macrophages or B cells that display the same antigen on their MHC-II by binding to the MHC-II/antigen complex and deliver co-stimulation through the surface receptors and cytokines. This can provide both B cell proliferation and activation of macrophages and sustains growth and differentiation of the T cell.

B cells have BCRs on their surface, secrete antibodies and cytokines and are professional antigen-binding cells. The activated B cell can further differentiate into plasmablasts, memory B cells and plasma cells. The BCR binds to antigens in their native form on pathogens, and the B cell, after activation, turn into a plasma cell that act by producing BCRs in a secreted form – the antibodies. Antibodies can neutralize or help eliminate pathogens. The BCR/antibody and the TCR are formed from different genes and have a distinct structure, but in both cases a similar genetic mechanism generates an incredible repertoire of diversity in the receptors. Each cell expresses one unique antigen receptor. Cells with autoreactive receptors are eliminated, and those with specificity to foreign antigens may be clonally expanded on demand. For B cells, a subsequent selection process ensures that there will be survival and expansion of cells in which the mutations have improved the antigen-binding affinity of the antibodies. Antibodies and details about the antibody – antigen binding will be described further in chapters 1.2.2 and 1.4.2.

The immune system has been most extensively studied in mammals, especially in mice and humans. Most features of the vertebrate adaptive immune system seem to be conserved trough evolution. However, there are still many differences between fish and mammals, and also between different fish species. Mammals are much younger and more homogeneous group of animals than fish, but even in mammals, there are some unusual differences in the immune system of some species (e.g. camelids lacking the light chain of their antibodies (Hamers-Casterman *et al.*, 1993), bovine antibodies possessing an especially long CDR3-loop (Stanfield *et al.*, 2016), rabbits and pigs relying on gene conversion instead of somatic recombination for creating antibody diversity (Tizard, 2013, Butler *et al.*, 2006, Knight & Barrington, 1998) and several species, including elephants lacking IgD

(Guo *et al.*, 2011)). Because of the greater diversity, there will likely be even more variations in the immune system in fish.

1.2.1 The salmonid immune system

The number of existing fish species where the immune system has been explored is microscopic compared to the high number of fish species. Over 40,000 species of bony fish exist and account for more than 50% of all known vertebrate species (Inoue *et al.*, 2010). Comparably, the number of mammalian species are only around 3500 (Burgin *et al.*, 2018). The bony fishes are divided into the ray-finned and the lobe-finned fishes. Among the ray-finned fishes we find the teleost fish, the largest group of living fishes, representing approximately 96% of bony fish species.

Fish inhabit vastly different ecosystems, from marine environments to fresh water, from the surface with only one to a few atmospheres pressure to the deepest oceans with a pressure almost 1000 times that of the surface, and from temperatures below zero to over 40°C. In addition, the chemical composition, pH and oxygen content in their environment can be extremely different. As fish are ectothermic, all chemical reactions and the time it takes to mount an immune reaction will be dependent on the temperature of the environment. Teleost fish vary in size from a few millimeters to several meters in length. They are the most primitive vertebrate group to possess an adaptive immune system and also the oldest group of animals. They are a much more diverse group than mammals. Considering this, caution must be used when extrapolating knowledge about one fish species to others. There are some known surprising features in the immune system of some fish species, for example cod (Star & Jentoft, 2012, Star *et al.*, 2011) and pipefish (Haase *et al.*, 2013) that lack MHC-II and CD4, catfish that have V-less secreted IgD (Edholm *et al.*, 2010) and the existence of a chimeric IgM/IgT antibody in the common carp (Savan *et al.*, 2005).

Atlantic salmon (*Salmo salar*) is a fish belonging to the teleost infraclass. Salmoninae is a sub-family of the family Salmonidae in the order Salmoniformes. It contains at least 126 species and includes the genera *Salmo* (47 species including Atlantic salmon and brown trout) and *Oncorhynchus* (15 species including rainbow trout). Atlantic salmon is a predominantly predatory carnivorous fish with an anadromous lifecycle. They hatch in freshwater where they live during their juvenile phase before they smoltify and migrate to the ocean. Here they stay until sexual maturation when they return to freshwater for spawning (Verspoor *et al.*, 2007). Salmonid fish are of great interest in comparative immunology due to their unique position in the phylogenetic tree and recent whole genome duplication event.

Farming conditions are exceedingly different from the natural life of Atlantic salmon. Examples of artificial conditions include confinement, high density rearing, monoculture of one species, excess food availability, artificial light regimes, reduced environmental variation, more plant-based feed, handling, rapid development and growth, absence of predation and artificial selection of breeding animals with focus on traits like rapid growth and delayed sexual maturation. These conditions may both inhibit the salmon's immune responses and increase virulence in pathogens. Exposing fish to chronic stress from for example crowding and handling can affect the immune response and increase the risk of infections (Tort, 2011, Pickering & Pottinger, 1989, Schreck *et al.*, 2016). Stress in early life of Atlantic salmon can cause lasting epigenetic changes in the genome, affecting immune responses. When studying the immune response to an LPS-challenge, fish that had experienced acute stress were found to have a similar but enhanced transcription profile of several types of immune genes. On the contrary, fish that had experienced chronic stress had a significant reduction of transcription of more than 200 genes, including a number of pro-inflammatory response markers (Uren Webster *et al.*, 2018b). Natural life events such as smoltification and sea water transfer also suppress most parts of the immune system (Johansson *et al.*, 2016). Gut microbiota can be different in wild and farmed Atlantic salmon (Uren Webster *et al.*, 2018a, Lavoie *et al.*, 2018), possibly due to differences in feed and early environment. The gut microbiota is important for the maturation of the immune system in mammals, but the possible impact of gut microbiota on the immune system of fish is unknown.

The following section will describe the salmonid/teleost immune system. The focus will be on B cells and the humoral part of the adaptive immune system. Where knowledge on Atlantic salmon is available this will be presented, otherwise information from rainbow trout (*Oncorhynchus mykiss*) or other salmonids or teleosts will be presented. A large part of the research on salmonid fish has been performed on rainbow trout. Although phylogenetically similar to Atlantic salmon, it is important to keep in mind that there could be more differences between the two species than what we know about at the moment.

Organization/organs

An obvious difference in fish compared to mammals is the lack of bone marrow and lymph nodes. The spleen is a secondary lymphoid organ in all vertebrates, and the thymus a primary lymphoid organ where T cells mature. In contrast to this, B cells mature in several different organs, bone marrow or ileal Peyer's patches in mammals, bursa of Fabricius in birds and head kidney in teleosts (Tizard, 2013). The head-kidney lacks nephrons and have no filtration or excretion function (Geven

& Klaren, 2017), but plays the role of hematopoietic organ with both erythropoiesis, myelopoiesis and lymphopoiesis (Zwollo *et al.*, 2005, Zwollo, 2018, Kobayashi *et al.*, 2016). In salmonids, long lived plasma cells are found in the head-kidney (Bromage *et al.*, 2004), and activation of B cells takes place in spleen and posterior kidney (Zwollo et al., 2005). Fish lack lymph nodes and germinal centers, but clusters of melanomacrophages are suggested to be analogues to the germinal centers found in lymph nodes in mammals (Magor, 2015, Haugarvoll *et al.*, 2006).

The mucosa-associated lymphoid tissues are important in fish. Skin, gut, gills and nostrils contain mucosa-associated lymphoid tissue (MALT) (Xu *et al.*, 2013, Haugarvoll *et al.*, 2008, Tacchi *et al.*, 2014) acting together with the spleen and posterior kidney as secondary lymphoid tissue (Parra *et al.*, 2013). Teleost skin lacks keratinization, so live cells are in direct contact with the environment. The skin contains mucus, skin-associated mucosal lymphoid tissue (SALT), IgT+ B cells and a microbiota coated with IgT (Xu et al., 2013). Other protective mechanisms of the mucosal surfaces include mucus shedding, antiviral and antibacterial peptides and antimicrobial enzymes. With all these functions, mucosal surfaces represent the first line of defense against pathogens (Salinas, 2015). The interbranchial lymphoid tissue (ILT), a macroscopically detectable structure, is found in the gills of salmonids and contain T cells (Aas *et al.*, 2014, Haugarvoll et al., 2008).

The innate immune system

The cellular part of the teleost innate immune system consists of macrophages, granulocytes (Havixbeck & Barreda, 2015), mast cells (Sfacteria *et al.*, 2015) and natural killer (NK) or NK-like cells (Nakanishi *et al.*, 2011). Teleost macrophages appear to function in the same way as the mammalian macrophages (Hodgkinson *et al.*, 2015), residing in most tissues where they patrol for, phagocytose and degrade pathogens. After degradation, the pathogen peptides can be presented to cells of the adaptive immune system on MHC-II. Melanomacrophages are a subpopulation of macrophages in ectothermic vertebrates. They are pigmented and participate in both immune defense and in physiological processes by phagocytosis of pathogens and exhausted cells, particularly erythrocytes (Steinel & Bolnick, 2017). Like in all vertebrates except mammals, fish erythrocytes are nucleated. They can therefore synthetize proteins, and express MHC-I on the surface as well as PRRs (Morera *et al.*, 2011, Dahle *et al.*, 2015, Sarder *et al.*, 2003, Dijkstra *et al.*, 2003). Both virus-infected and non-infected salmon erythrocytes can produce interferon and anti-viral proteins (Morera et al., 2011, Nombela & Ortega-Villaizan, 2018, Dahle et al., 2015, Haatveit *et al.*, 2017). Rainbow trout erythrocytes can phagocytose pathogens (Passantino *et al.*, 2002) and respond to the double-stranded RNA (dsRNA) mimic Poly(I:C) by secreting molecules that can modulate an anti-viral response in macrophages (Morera et al., 2011). Teleost thrombocytes are also nucleated. In addition

to being functionally equivalent to the anucleated mammalian platelets, they can phagocytose and kill bacteria (Nagasawa *et al.*, 2014).

Several types of molecules are parts of the innate humoral immune system. Antimicrobial peptides (AMPs) are small proteins conserved in plants, invertebrates and vertebrates. In fish, they are produced by several cell types, primarily at mucosal surfaces. They have a broad-spectrum antimicrobial activity against bacteria, virus, fungi and protozoa, and an immunomodulatory function. Cathelicidins, hepcidins, histone-derived peptides, defensins and piscidins are AMPs described in teleosts (Masso-Silva & Diamond, 2014). Cytokines regulate cellular functions like proliferation, inflammation and the immune response, and function as communication between the innate and the adaptive immune system. Interferons are virus-induced cytokines that can regulate the expression of several hundred genes, particularly antiviral proteins. Several interferon genes have been cloned from Atlantic salmon (Robertsen *et al.*, 2003). The cytokine repertoire in teleosts is generally similar to what is found in mammals, however, the sequence identities are low and the functions largely unexplored compared to their mammalian counterparts (Whyte, 2007). Complement is an ancient mechanism also present in teleosts. Complement of cold water teleosts have a lower temperature optimum than mammalian complement (Boshra *et al.*, 2006).

The adaptive immune system

The adaptive immune system of fish is, like in other vertebrates, based on antigen recognition by immunoglobulins and T-cell receptors, and on antigen presentation by MHC molecules. There are however many similarities between mammalian innate-like lymphocytes and teleost B and T cells (Scapigliati *et al.*, 2018). Salmonids will typically be around 0,5-1 g when they are fully immunocompetent and able to mount an adaptive response (Tatner & Horne, 1983).

All of the TCR chains found in mammals (α, β, γ and δ) (Rast *et al.*, 1995, Partula *et al.*, 1995, Imai *et al.*, 2005) and the co-receptors CD8 and CD4 (Hansen & Strassburger, 2000, Laing *et al.*, 2006) have been found in teleost fish. The main subpopulations of CD4+ T cells: the T_h1 cell, T_h2 cell, T_h17 cell (Wang & Secombes, 2013) and perhaps T_h22 (Hu *et al.*, 2019) seem to be present in fish, but have not been functionally characterized. In contrast to in mammals, MHC-I and MHC-II are located on different chromosomes in fish (Wegner, 2008).

Teleost B-cells are the antibody-producing cells and their purpose is to produce antibodies that can bind to foreign molecules and pathogens entering the body (Ye *et al.*, 2013, Zwollo, 2018). The antibody produced by the B cells is a secreted form of the BCR, identical to this B cell's BCR except in

the C terminal end. The membrane bound C terminal of the BCR has a long string of hydrophobic amino acids that traverses the lipid bilayer of the cell membrane. The secreted form instead has a shorter string of hydrophilic amino acids. A B cell express around 10^5 BCRs on its surface. Each BCR has two associated intracellular signaling molecules, Igα and Igβ, connecting it to intracellular signaling pathways (Hombach *et al.*, 1990). Antigen presentation, phagocytosis and production of polyreactive antibodies are other functions of B cells in teleost fish (Zhu *et al.*, 2014, Li *et al.*, 2006, Sinyakov *et al.*, 2002), but it is not known if the different functions are performed by different subsets of B cells or not. B cells are dependent on stimulation from T_h cells to produce antibodies against protein antigens (T-dependent antigens). Polysaccharides, glycoproteins, lipids and nucleic acids can stimulate B cells to produce antibodies in a T-independent manner (Abbas et al., 2015). B cells in teleosts are suggested to be more related to mammalian B1 cells than B2 cells, based on functions and the expression of the B1 cell marker CD5 (Abos *et al.*, 2018a, Sunyer, 2012, Zhang *et al.*, 2017) and PRRs (Abos *et al.*, 2013, Abos *et al.*, 2015), and the ability to produce B cell-activating factor (BAFF) (Tafalla *et al.*, 2017). In mammals, B1 cells are usually classified as part of the innate immune system (Rothstein *et al.*, 2013) and produce most of the so called natural antibodies in serum (Baumgarth *et al.*, 1999). The natural antibodies are present before contact with antigens or triggered by contact with an antigen mainly through a T cell-independent pathway. In general subpopulations and differentiation stages in teleost B cells are largely unknown due to a lack of identified surface markers and tools to characterize them.

1.2.2 Antibodies

Structure

Antibodies, or immunoglobulins, exist only in jawed vertebrates and are one of the most important molecules in the adaptive immune system. Immunoglobulins are heterodimeric glycoproteins formed by four polypeptide chains, two identical heavy chains (H) and two identical light chains (L) (figure 2). The chains are held together by disulfide bridges, and antibody monomers of some isotypes can form antibody polymers. The antibody isotype is determined by the heavy chain. The heavy and light chains are encoded by the IgH and IgL locus in the genome respectively. All immunoglobulin molecules contain two or more carbohydrate chains, usually linked to the heavy chains. An antibody monomer is often imagined shaped like a Y. The two arms are denoted Fab (Fragment antigen-binding), and the stem Fc (Fragment crystallizable) based on chemical properties (figure 2). The Fab part contains the antigen binding sites, the paratopes, located at the end of both arms. Here parts of the heavy and light chain together form a groove where the antigen epitope can

fit. The Fc portion is responsible for the antibody effector function and contact with the rest of the immune system via Fc receptors expressed on other cells of the immune system. This way cells like macrophages can detect pathogens covered with bound antibodies or the complement cascade can be activated. Glycosylation of the antibody Fc region can affect function, structure, binding, solubility, conformation and subcellular transport and secretion (Arnold *et al.*, 2007). Antibodies can act in several different ways, including: directly block the function of a pathogen (neutralizing antibody), help in the internalization of a pathogen by a phagocytic cell (opsonizing antibody) and enable antigen-dependent cellular cytotoxicity.

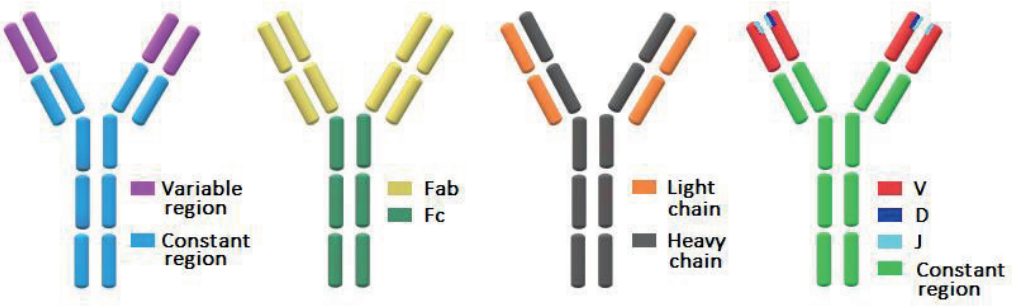

Figure 2. *Schematic presentation of an antibody monomer with different regions*

The antigen binding site, called the paratope is made up of the heavy chain and light chain variable domains (Danilova & Amemiya, 2009). The diversity in the amino acid sequence in both the heavy and light chain are concentrated in regions called complementarity-determining regions (CDRs) which make up the paratope. Each variable light domain and each variable heavy domain contains three CDRs (CDR1, CDR2 and CDR3). The CDRs are situated apart from each other in the amino acid sequence but are brought together when the protein is folded (figure 3). Between the CDRs are four less variable framework regions largely made up of anti-parallel β-sheets.

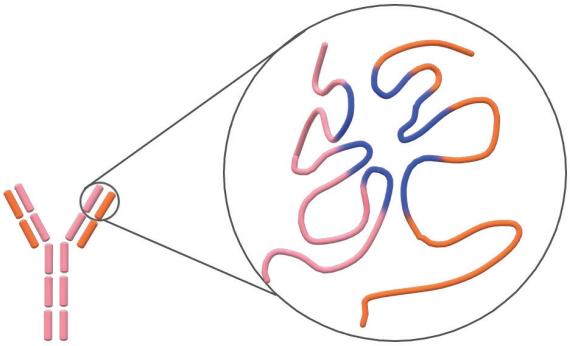

Figure 3. *Simplified illustration of the antibody paratope made up of the CDRs in blue.*

Antibody types in fish

Three immunoglobulin classes are found in Atlantic salmon: IgM, IgD and IgT (Hikima *et al.*, 2011). The heavy chains of teleost antibodies are either made up of several μ, δ or τ constant domains, making IgM, IgD or IgT respectively. In addition to the constant domains, there is one variable domain encoded by the V, D and J gene segments. The V, D, J and constant (C) gene segments are located in tandem arrays, denoted $(VH)_n$-$(D)_m$-$(JH)_x$-$(CH)_y$, also known as a translocon organization (Hordvik *et al.*, 1997, Hikima et al., 2011, Fillatreau *et al.*, 2013). The C_τ genes of Atlantic salmon are scattered in the VH region upstream of the Cμ and Cδ genes.

The IgL locus lacks the D segment, and the gene segments encoding the light chain are organized in clusters in teleost, $(V\text{-}J\text{-}C)_n$ (Cannon *et al.*, 2004, Criscitiello & Flajnik, 2007). This organization is opposed to the translocon organization of the heavy chain. The cluster organization of Ig chains are also found in cartilaginous fish, in both the heavy and light chain, whereas in mammals both the heavy and light chain have a translocon organization. Four IgL isotypes have been described in teleost fish, κF, κG, σ and λ (Edholm *et al.*, 2011). Three IgL isotypes found in salmon have been called L1, L2 and L3 (Solem & Jorgensen, 2002). In rainbow trout eight IgL isotypes were identified. L1 and two others were homologous to IgLκG, L3 and one other were homologous to IgLκF, one was homologous to IgLλ, and L2 and one other were homologous to IgLσ (Zhang *et al.*, 2016). All isotypes of rainbow trout participated in an immune reaction stimulated by Poly(I:C) or LPS. IgLκG was dominating in most tissues except in mucosa-associated lymphoid tissues and in serum IgT where IgLσ dominated (Zhang et al., 2016).

All teleost fish have gone through three whole genome duplication events, two more than 500 million years ago, before the divergence of lampreys from jawed vertebrates (Smith *et al.*, 2013), and one around 320 million years ago (Nakatani *et al.*, 2007). A fourth round of whole gnome duplication occurred between 125 and 80 million years ago in the common salmonid ancestor (Lien *et al.*, 2016). Due to this last duplication Atlantic salmon possesses two paralogous Ig heavy chain gene complexes with three functional Cτ genes, two Cμ genes and two Cδ genes (Yasuike *et al.*, 2010) located on separate chromosomes. Therefore, Atlantic salmon possess two IgM isotypes, IgM-A and IgM-B (and two IgD genes, three intact IgT genes and five IgT pseudogenes), leading to one of the largest potential combinatorial Ab repertoire of any vertebrates (Hordvik *et al.*, 2002, Yasuike et al., 2010). The IgM-A: IgM-B ratio in serum and the ratio of IgM-A: IgM-B displaying lymphocytes is typically 40:60, although this can depend on infection status (Kamil *et al.*, 2013a, Hedfors *et al.*, 2012).

IgM is the dominant isotype in teleosts and exists as a membrane bound and a secretory form. It is found as a tetramer in serum (Hordvik, 2015, Acton *et al.*, 1971). Teleost IgM is considered to be both structurally and functionally homologous to mammalian IgM. Mammalian IgM is of low affinity compared to the mammalian IgG (Notkins, 2004), but, because of its polyvalence, IgM has a high avidity. IgM contributes to both innate and adaptive immunity in fish. IgM effector functions include complement activation which can both lyses or opsonizes pathogens (Boshra *et al.*, 2004, Cooper, 1985). IgM also mediates agglutination for phagocytosis and pathogen removal, and cellular cytotoxicity (Ye et al., 2013). Serum IgM concentrations have been found to be between 800 and 9000 μg/mL in teleosts (Ye et al., 2013), and from 800 - 1300 μg/ml in Atlantic salmon (Havarstein *et al.*, 1988, Hordvik, 2015). The half-life of serum IgM is 1-3 days in salmonids (Voss *et al.*, 1980, Ye *et al.*, 2010). The IgM concentration in rainbow trout serum has been measured to between 100 and 1000 times as high as the concentration of IgT (Zhang *et al.*, 2010, Castro *et al.*, 2013) and transcript levels of μ chains in Atlantic salmon is more than 200 times that of δ chains. IgM can also be found in mucus (Zhang et al., 2010, Xu et al., 2013, Xu *et al.*, 2016), bile (Lobb & Clem, 1981) and eggs (Swain & Nayak, 2009). The polymeric Immunoglobulin receptor (PIgR) transfers IgM to the mucus (Zhang et al., 2010) or IgM can be produced locally (Zhao *et al.*, 2008). The secreted form of IgM consists of μ chains made from four constant domains (μ1- μ2- μ3-μ4), and the membrane bound form of only three constant domains, but with a transmembrane part (μ1- μ2- μ3-TM) at the C-terminal (Hordvik *et al.*, 1992). IgLκG is the light chain isotype most often expressed with the heavy chains of IgM in rainbow trout (Zhang et al., 2016). In rainbow trout, B cells expressing membrane-bound IgM are also found to be IgD+ and IgT-.

In other vertebrates, IgM is a pentamer, and a J chain (a polypeptide, not to be confused with the J gene segment) as well as disulfide cross linking is involved in the polymerization. In teleosts, no J-chain homologue has been found, and the monomers seem to be more loosely linked by disulfide binding (Klimovich *et al.*, 2008). The degree of disulfide binding between the monomers has been shown to correlate with increased antibody affinity in rainbow trout and the fraction of fully cross-linked IgM increased during the immune response (Ye et al., 2010, Ye *et al.*, 2011a, Ye et al., 2013). The IgM-B of Atlantic salmon seems to have a higher degree of disulfide binding than IgM-A (Kamil *et al.*, 2013b), possibly because of an extra cysteine residue close to the C-terminal part of the IgM-B (Hordvik et al., 2002).

Teleost IgD is a chimeric molecule consisting of one Cμ1 domain and a number of Cδ domains depending on the species. Mammalian IgD is not chimeric, and contains only Cδ domains, except in some artiodactyl species (Zhao *et al.*, 2003). Rainbow trout IgD contains six Cδ domains (Ramirez-Gomez *et al.*, 2012). The δ gene is located immediately downstream of the μ gene and both IgM and IgD can be expressed in the same lymphocyte. This is regulated by alternative splicing, a mechanism of post-transcriptional processing of the primary-mRNA (pre-mRNA). By removing or retaining introns and including exons in different combinations, a large proteome can be achieved from a limited number of genes (Nilsen & Graveley, 2010, Keren *et al.*, 2010). The IgD concentration in serum is 2–80 μg/ml. IgD secreting cells can be found in anterior and posterior kidneys, spleen and gills (Bengten & Wilson, 2015). Functions of IgD are largely unknown both in teleosts and mammals, but it has been suggested that it binds to an unknown Fc receptor on basophils and are involved in inflammatory responses (Chen *et al.*, 2009, Edholm et al., 2010). An IgD+/IgM- B cell population has been described in rainbow trout (Castro *et al.*, 2014a).

In salmonids, IgT is a monomer in blood and a tetramer in mucus. Rainbow trout IgT has four C domains (Salinas *et al.*, 2011). It is considered to be specialized to mucosal immune responses, analogous to mammalian IgA (Tadiso *et al.*, 2011, Zhang et al., 2010, Xu et al., 2013, Sunyer, 2013, Gomez *et al.*, 2013), but can also have an important role outside the mucosa. IgT responses in spleen have been reported after intramuscular injection with attenuated viral hemorrhagic septicemia virus (VHSV) (Castro et al., 2013) and in muscle tissue after vaccination with a DNA vaccine against the same virus (Castro *et al.*, 2014b). IgT + B cells do not express the other two immunoglobulin types (Hansen *et al.*, 2005, Salinas et al., 2011).

Diversity

The diversity of BCRs and antibodies is immense; they can bind proteins, carbohydrates, lipids and nucleic acids. Each antibody molecule is specific, and an antibody can distinguish between a linear protein epitope where only one amino acid is substituted even if the secondary structure is unchanged. This ensures that an antibody towards a pathogen cannot bind to self-antigens. However, some antibodies may cross-react, meaning that they can bind to different, but structurally related antigens. This antibody diversity could not be possible using only germ line normal transcription, as that would mean that a disproportionately large fraction of the genome would be immunoglobulin genes. Susumu Tonegawa was awarded the Nobel prize in medicine in 1987 for the discovery of the genetic mechanism behind this diversity (Tonegawa, 1993). In all cells except the B lymphocytes, the genes encoding the variable and constant domains of the BCR are separated by a considerable distance in the genome. Early in B cell development, in primary lymphoid organs, these gene segments are brought together through a process called somatic recombination (figure 4). It is a random rearrangement of the variable-(diversity)-joining (V-(D)-J) gene segments mediated by the recombination-activating gene products (RAG-1 and RAG-2), expressed only by lymphocytes (Bassing *et al.*, 2002, Schlissel, 2003, Jung *et al.*, 2006, Parra et al., 2013). Somatic recombination results in the generation of diverse B (and T) cell receptors capable of recognizing a large variety of pathogens (Danilova & Amemiya, 2009, Schatz *et al.*, 1992, Neuberger, 2008). The theoretical diversity of the antibody repertoire after recombination is estimated from 10^{12} to 10^{18} in humans. The actual repertoire of possible functional antibodies is much smaller (Elhanati *et al.*, 2015, Weigert *et al.*, 1980).

Additional diversity is introduced during random assortment of the H and L chains during which any H chain may associate with any L chain. Inaccuracies occur at the precise positions where the V, D and J segments join, resulting in junctional diversity. Small sets of nucleotides can also be inserted at the V-D and D-J junctions. This is mediated by the enzyme terminal deoxynucleotidyl transferase (TdT). Together all this leads to a high number of possible receptors. All this takes place in immature B cells giving each B-cell clone only one unique antibody specificity. The rearrangement is independent of antigens, and is the organism's pre-made defense before contact with pathogens. Therefore, the affinity of these pre-made antibodies is generally lower than those made later, during an infection.

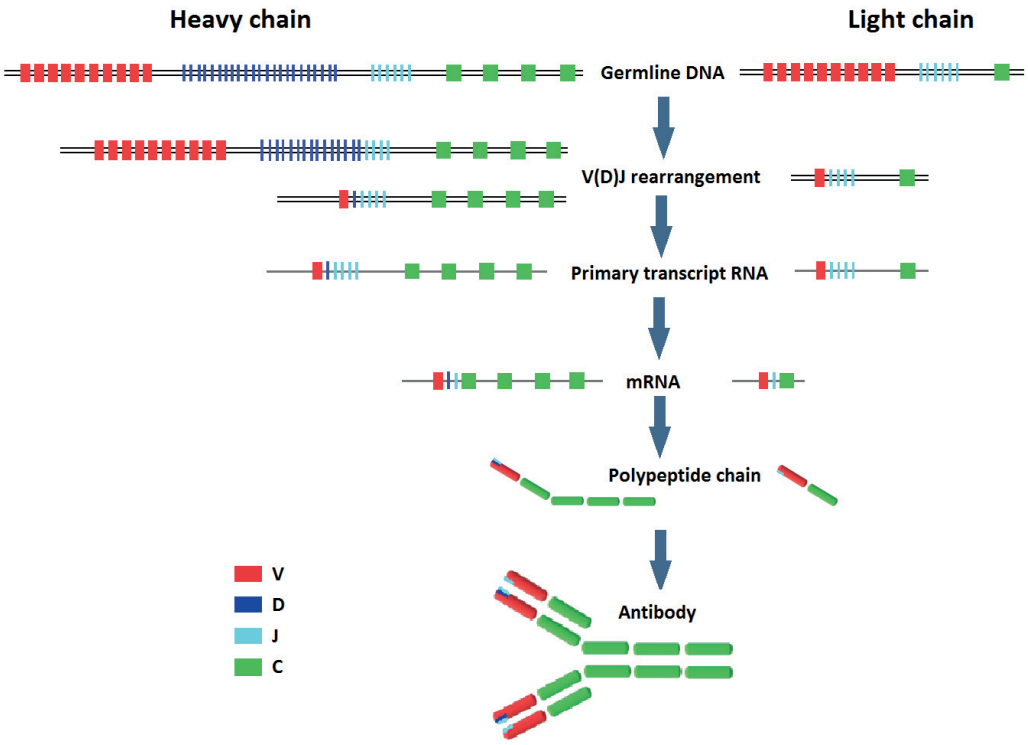

Figure 4. *Somatic recombination and construction of immunoglobulin heavy and light chains.*

In mammals, B cells and T_h cells meet in lymphoid follicles together with follicular dendritic cells and form germinal centers where B cells proliferate and undergo somatic hypermutation. This is a fine tuning of the specificity of the V regions, initiated by the enzyme activation-induced cytidine deaminase (AID), where both the light and heavy chain undergo a high rate of point mutations. The mutations are concentrated in the CDRs. AID converts a cytosine (C) to uracil (U) at specific locations in the variable genes of the heavy and light chains. The DNA repair machinery in the cell removes the U as it is regarded an error, and then, through different mechanisms, a new base is inserted. The repair machinery is error-prone, and the result is at least ten-fold higher mutation rate than normal. The daughter cells with their different point mutations, and thereby different antigen affinities, compete to bind antigens presented by follicular dendritic cells (FDC). The amount of antigen is limited, meaning that only the B cells with the highest affinity BCR can bind. Only the most successful cells are “saved” from pre-programmed apoptosis by T_h cells and undergo clonal expansion before they differentiate into either antibody producing plasma cells or memory B-cells. This affinity maturation takes place in secondary lymphoid organs and goes on over several rounds. In mammals,

the new antibodies can have a 10 to 5000 fold higher affinity than the antibodies produced by the original B cell (Mishra & Mariuzza, 2018). The changes made in the genome by somatic recombination and somatic hypermutation are permanent changes in each B cell. The structural basis of affinity maturation seems to be a decrease in the conformational flexibility of the antigen binding site of the antibody (Manivel *et al.*, 2000, Di Palma & Tramontano, 2017).

Mammals have the ability of class switching or isotype switching, meaning that after a successful recombination, they can re-arrange the constant region genes so that the variable gene region can be expressed with a different constant region. The new antibody will have the same antigen specificity, but a different effector function. The isotype switching is mediated by AID and is a region-specific recombination that replaces the exons encoding the μ chain (Cμ) of the Ig heavy chain (IgH) with a downstream (3') constant region exon (CH) cluster such as Cγ or Cα. Mammalian B cells can therefore change from expressing the isotype IgM to isotypes with different effector functions, such as IgG or IgA. Isotype switching is present in amphibians, but not in fish. Still, teleost AID can mediate class switching in mouse cell lines (Barreto & Magor, 2011, Wakae *et al.*, 2006). The absence of class switching in fish may be related to an absence of an appropriately organized immunoglobulin gene. The recombination takes place in a specific S-region that has not been found in teleost IgH loci, and there are no additional CH genes located 3' of Cμ and Cδ. The τ genes are located between variable gene segments upstream (5') of the μ and δ genes, possessing its own D and JH segments (Yasuike et al., 2010), and are possible deleted upon IgM rearrangement (Danilova *et al.*, 2005, Hansen et al., 2005).

Diversity and affinity maturation in fish?

It was first believed that no somatic hypermutation or affinity maturation occurred in fish due to the lack of germinal centers and absence of high affinity immunoglobulins (Magor, 2015). In addition, somatic hypermutation mainly takes place after the B cell has switched from producing IgM to other isotypes in mammals (Kitaura *et al.*, 2017). Somatic hypermutation does however exists in amphibians (Wilson *et al.*, 1992) and sharks (Diaz *et al.*, 1999, Diaz *et al.*, 1998), and it is therefore likely to also be present in teleosts. AID from fugu, zebrafish and channel catfish have been shown to induce mutations when expressed in bacteria, yeast and mouse cell lines (Wakae et al., 2006, Barreto *et al.*, 2005). It has also been demonstrated that mammalian B1 cells accumulate somatic hypermutations as they age (Yang *et al.*, 2015), that this is AID-dependent, and that somatic hypermutation also takes place in human fetal B cells in a T-independent fashion (Rechavi *et al.*, 2015). The gene encoding TdT has been found in rainbow trout (Hansen, 1997). RAG1 and RAG2 from

fish were first discovered in rainbow trout (Hansen & Kaattari, 1996). In rainbow trout, RAG is expressed in cells from around 10 days after fertilization, and IgM from around hatching (Razquin *et al.*, 1990) or approximately 3 weeks after fertilization (Hansen, 1997). A study on rainbow trout (Cain *et al.*, 2002) revealed that higher affinity IgM antibodies occurred after immunization with a T-dependent antigen. The affinity increased 2-3-fold during the 12 weeks of the study. This increase is of a much lower magnitude than what can be observed in mammals. Another study in rainbow trout has also shown that low affinity IgM was present early after infection, while high affinity IgM developed later during the immune response (Ye et al., 2010, Kaattari *et al.*, 1998).

There are no lymph nodes or germinal centers in fish, and the microenvironment needed for affinity maturation seems to be lacking. The slow increases in affinity must therefore be achieved through alternative mechanisms. Several studies on affinity maturation in human autoimmune disorders have noted that loose and often ectopic aggregates of B- and T-cells, with or without associated FCDs, can generate antigen selected B-cells (Schroder *et al.*, 1996, William *et al.*, 2002, Da *et al.*, 2007). Clusters of melanomacrophages are observed in kidney, liver and spleen in several teleost fish species, as well as in amphibians and reptiles. Melanomacrophages reside mainly in spleen, kidney and inflamed tissues, have been found to accumulate at sites of long-term antigen retention in salmonids (Agius & Roberts, 2003, Koppang *et al.*, 2005, Espenes *et al.*, 1995) and may act as FDCs in mammals presenting antigens to B-cells (Saunders *et al.*, 2010). Melanomacrophage centers (MMCs) are surrounded by a reticular cell stroma (Herraez & Zapata, 1991, Press *et al.*, 1994, Diaz-Satizabal & Magor, 2015). These reticular cells could also be a candidate for antigen trapping as they are related to FDCs (Jarjour *et al.*, 2014). Antigens can be retained for months on or close to the MMCs in fish, and was found to be located extracellularly at the membrane of cells in and around the melanomacrophage centers for at least a year in carp injected with *Aeromonas hydrophila* (Lamers & De Haas, 1985). The MMCs increase in size and/or numbers following both infections and non-infectious events like starvation, pollution, injury and aging (Steinel & Bolnick, 2017). Lymphocyte-like cells have also been observed at the MMCs (Falk & Dannevig, 1995, Fournier-Betz *et al.*, 2000), and it has been hypothesized that the MMCs are analogous to germinal centers in mammals. A clear difference between germinal centers and MMCs are the amount of antigen that is far greater in MMC. By analyzing gene expression in microdissected cell subsets from histological sections of channel catfish tissues it was found that IgH, TCRβ and CD4 were expressed in both leukocyte rich regions and MMCs, but that AID was only expressed in MMCs (Saunders et al., 2010). The AID expressing cells are far outnumbered by antigen-trapping cells (melanomacrophages or reticular cells), meaning that all B cells that can bind the antigen will be selected for survival and clonal expansion. This is in contrast to the germinal centers in mammals where only the B cells with the

highest affinity can bind to the limited amount of antigen. This leads to more antigen producing cells in fish producing antibodies with much lower affinity than in mammals. Not selecting away any B cells that can bind the antigen could mean a faster antibody response, and as IgM is tetrameric, the lower affinity can be made up for by the higher avidity. However, during the course of an infection, as the number of pathogens decrease, there could be less antigen available for binding in the MMC. This could lead to more competition between B cells with different affinities and a selection similar to what is seen in mammals.

A study on rainbow trout looked at the distribution of various CDR3 lengths for IgM-, IgT- and IgD-expressing cells in the spleen (Castro et al., 2013). In healthy fish, the CDR3 lengths for all isotypes had a normal distribution. For VHSV-infected fish, peaks were demonstrated for IgM and IgT, but not for IgD. Some peaks were found in only one fish, others in almost all. This suggests that both IgM+ and IgT+ B cells in the spleen responds to the virus with characteristic clonal expansions.

Yang et al analyzed the distribution of replacement mutations and silent mutations in the framework and CDRs in catfish B cells. They concluded that there was no evidence for selection of B-cells with an altered BCR. They observed evidence of clonal lineage development but concluded that the mutations were generated as part of the primary antibody development (Yang *et al.*, 2006).

It has been observed that high affinity antibodies have a longer half-life than antibodies of lower affinity. An explanation for this has been proposed by Ye at al (Ye et al., 2010). The mechanism proposed is that if a BCR binds an antigen with high affinity, the antibody produced by this B cell will be post-translationally modified to a greater extent than if the antigen-BCR binding is of low affinity. The post-translational changes are mainly disulfide polymerization, which could limit recognition of the secreted Ab by receptors such as the PIgR, a receptor which would otherwise quickly remove the antibody from circulation. If this is the case, it could contribute to the findings that antibody affinity increases during the weeks after infection. However, the half-life of IgM in fish is only a few days, this cannot be the whole explanation. The difference in modification can also affect the effector function of the antibody. A B cell that binds an antigen strongly with its BCR will therefore produce an antibody with a different effector function than if it binds an antigen more weakly (Ye et al., 2013). This might be an alternative in teleosts to the isotype switching in mammals.

1.2.3 Polyreactive antibodies

Polyreactive antibodies are defined as antibodies capable of binding two or more unrelated antigens. B1 cells in mammals produce polyreactive antibodies called natural antibodies (Zhou *et al.*, 2007). They can develop in the host after exposure to an antigen or can be germline-encoded and present

before contact with antigens (New *et al.*, 2016). There is much confusion on the definition and terminology when it comes to antibodies that are not the classical type produced by mammalian B2 cells in germinal centers after contact with antigen. Different publications talk about natural antibodies, polyreactive antibodies, non-specific antibodies, natural autoantibodies, pre-existing antibodies, innate antibodies and background antibodies. The definitions are ambiguous, and it is not always clear if they are talking about the same or of different types of antibodies. Strictly speaking, one should demonstrate multiple binding capacities to call an antibody polyreactive and use germ-free animals to prove natural antibodies present before contact with antigen. So-called natural antibodies are found in serum of fish and other vertebrates, but this has often been dismissed as background noise in serological assays. In mammals, they are often bound to self-antigen (Notkins, 2004, Casali & Notkins, 1989), can facilitate complement-mediated neutralization (Jayasekera *et al.*, 2007) and when binding to apoptotic cells, lead to fixation of complement and increased phagocytosis by macrophages (Gunti & Notkins, 2015). They also protect against pathogens and bind molecules associated with neurodegenerative diseases, tumors and atherosclerosis in mammals (Palma *et al.*, 2018). They can be of different isotypes, are germline or near germline, already rearranged and develop before birth and before contact with an antigen (Merbl *et al.*, 2007) and exhibit a structural flexibility (Zhou et al., 2007, Notkins, 2004, James *et al.*, 2003). This flexibility is thought to be the reason they can bind different epitopes (figure 5).

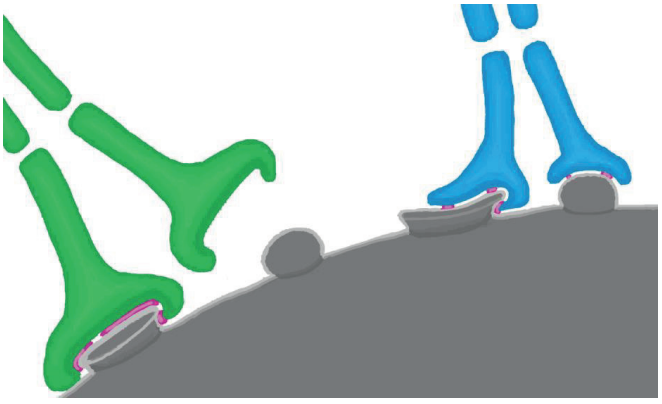

Figure 5. *A specific antibody (left) binding with high affinity (symbolized with pink color) to one specific epitope and a polyreactive antibody with lower affinity and flexible binding seat (right) binding a smaller part of two different epitopes. It can still bind as it has two binding sites (or eight if IgM) and therefore high avidity.*

Natural antibodies should be distinguished from the apparent polyreactivity, a property of polyclonal serum occurring via non-antigen-specific antibody production, often in response to stimulation of B-cells by for example LPS (Montes *et al.*, 2007). Trinitrophenol (TNP), dinitrophenol (DNP) or DNP-keyhole limpet hemocyanin (DNP-KLH) has been used to measure the level of these or polyreactive antibodies (Gunti & Notkins, 2015, Lund *et al.*, 2019). Anti-TNP antibodies can be found in phylogenetically distant species of vertebrates. When anti-TNP antibodies were isolated from unimmunized fish of several species, they were found to be highly cross-reactive, and could, in addition to TNP-KLH, bind to actin, myosin, thyroglobulin and single-stranded DNA (Gonzalez *et al.*, 1988). This cross-binding was strongest in sharks and sturgeons, and much weaker in teleosts. The authors suggest that this could be because so-called natural antibodies are more important in more primitive fishes.

The antibody repertoire studied with high-throughput sequencing in zebrafish looked at the use of possible VDJμ combinations from 51 fish (Jiang *et al.*, 2011). This showed that at two weeks post fertilization, only 20 % of the possible combinations were used, and that a few combinations dominated and were shared among individuals. At two months, this had changed. Now around 70% of the possible combinations were in use, and the dominant shared combinations were no longer observed. This suggests that the antibodies produced first are “natural antibodies” developmentally controlled, and that the change from two months most likely is due to clonal expansion of activated B cells expressing their unique combination in response to environmental antigens.

In this work, the term polyreactive antibodies is used for non-specific antibodies seen after PRV-infection. It is not clear, however, if what we have observed is truly polyreactive antibodies, natural antibodies or a polyclonal antibody response with non-specific antibodies, or a combination of two or more of these.

1.2.4 B cell development and B cell memory

The common lymphocyte progenitor cell is the first development stage of lymphoid cells. In rainbow trout, RAG is expressed in cells from around 10 days after fertilization, and IgM from around hatching (Razquin et al., 1990) or approximately 3 weeks after fertilization (Hansen, 1997). Development of the teleost B cells goes through several subsequent stages: pro-B cell, pre-B cell, immature B cell and mature B cell. (Zwollo et al., 2005, Zwollo, 2011). Naïve B cells are IgD+IgM+ (Abbas et al., 2015). In spleen and posterior kidney, they are activated by APCs. Mature naïve B cells in head kidney migrate to blood, spleen and posterior kidney. In mammals, activated B cells are IgD-IgM+ and also larger in size compared to naïve B cells (Parham & Janeway, 2015). Activated B cells can develop into short-

lived plasma cells in spleen or to low antibody secreting plasmablasts with low levels of membrane bound immunoglobulin. Plasmablasts that migrate to the head kidney can develop into non-replicating high antibody secreting long-lived plasma cells. Plasma cells do not have membrane-bound immunoglobulin (Parham & Janeway, 2015). A population of IgD+IgM- cells have been identified in rainbow trout gills (Castro et al., 2014a) and kidney (Abos *et al.*, 2018b). Both IgM+ and IgT+ B cells are recruited to inflammation sites, and can differentiate locally to antibody-secreting cells or plasmablasts in response to virus (Castro *et al.*, 2017, Castro et al., 2014b).

A fundamental concept of the adaptive immune system is the ability to "remember" past encounters with pathogens, or more precisely with antigens. The ability to mount a faster and stronger as well as specific response at subsequent encounters are called immunological memory. In mammals, the secondary responses are exactly that, faster and with higher levels of specific antibodies with increased affinity. In mammals, a B cell can be stimulated by T cells to express bcl-2, which facilitates its survival and differentiation into a memory B cell. There are two subsets of memory B cells, one type is resting small cells that are IgG+ and not dependent on antigen contact for survival, the other type is large, dividing IgM+ cells that are dependent on stimulation with antigen to survive. Memory B cells can survive for up to 60 years after vaccination in humans. B1 cells of mammals can also generate memory B cells (Kurosaki *et al.*, 2015, Yang *et al.*, 2012) .

In contrast to the antibody responses seen in mammals, the primary antibody response seems to be slower and of longer duration in fish. A prolonged primary response might be responsible for protection after vaccination or infection in some cases (Yamaguchi *et al.*, 2018). Still a secondary response occurring faster and with higher titres of antibodies has been demonstrated in rainbow trout (Cossarini-Dunier, 1986, Tatner *et al.*, 1987, Ma *et al.*, 2013). A DNA vaccine against infectious hematopoietic necrosis (IHN) has been shown to offer protection for two years following vaccination of rainbow trout (Kurath *et al.*, 2006). Another DNA vaccine also showed long-term protection against viral hemorrhagic septicemia virus (VHSV) in rainbow trout (Lorenzen *et al.*, 2002). However, this is not necessarily a secondary response, but could be caused by a continuous expression of injected plasmid proteins as the plasmids can be detected for longer periods (Tonheim *et al.*, 2007, Dijkstra *et al.*, 2001). A study with repeated bath vaccinations followed by a challenge with *Yersinia ruckeri* in rainbow trout failed to find any evidence of a secondary immune response (Jaafar *et al.*, 2018). However, salmon surviving PD are less susceptible to a second SAV infection at least up to 9 months after initial infection (Houghton, 1994) indicating memory and a secondary response or a very long primary response.

Plasmablasts can differentiate into either memory cells or long-lived or short-lived plasma cells. After immunization, a large pool of long-lived plasma cells is present in the anterior kidney (Ma et al., 2013, Bromage et al., 2004, Kaattari *et al.*, 2005). Resting, non-Ig-secreting cells that could be memory cells are found in blood (Ma et al., 2013, Ye et al., 2013), but fish memory cells are poorly defined, and their existence remains to be verified.

1.3 THE VIRUSES AND DISEASES

Viral diseases represent a huge challenge in aquaculture. Pancreas disease (PD) caused by Salmonid alphavirus (SAV) and heart and skeletal muscle inflammation (HSMI) caused by *Piscine orthoreovirus* (PRV) are two important viral infections in Norwegian aquaculture. Details of the viruses are listed in table 1. Two subtypes of SAV cause disease in Norway, SAV2 at the coast in mid-Norway and SAV3 at the south-western coast. PD is the most frequent notifiable disease in Norwegian aquaculture with 163 registered outbreaks in Norway in 2018, 98 caused by SAV3 and 63 by SAV2. PRV is ubiquitous in Norwegian aquaculture. HSMI was detected at 104 localities by the Norwegian Veterinary Institute in 2018 but as it is not a notifiable disease, the numbers are underreported. In addition, private laboratories detected HSMI at 90 localities, but these numbers may be partly overlapping (Norwegian Veterinary Institute, 2019). Both diseases decrease the salmon's ability to handle stress, which could be an underlying cause of mortality associated with sea lice treatment or other stressful events. Both PD and HSMI are major economical and welfare problem for the salmon farming industry. Figure 6 shows the life cycle of farmed Atlantic salmon with times of vaccination, smoltification, sea transfer and common periods of HSMI and PD.

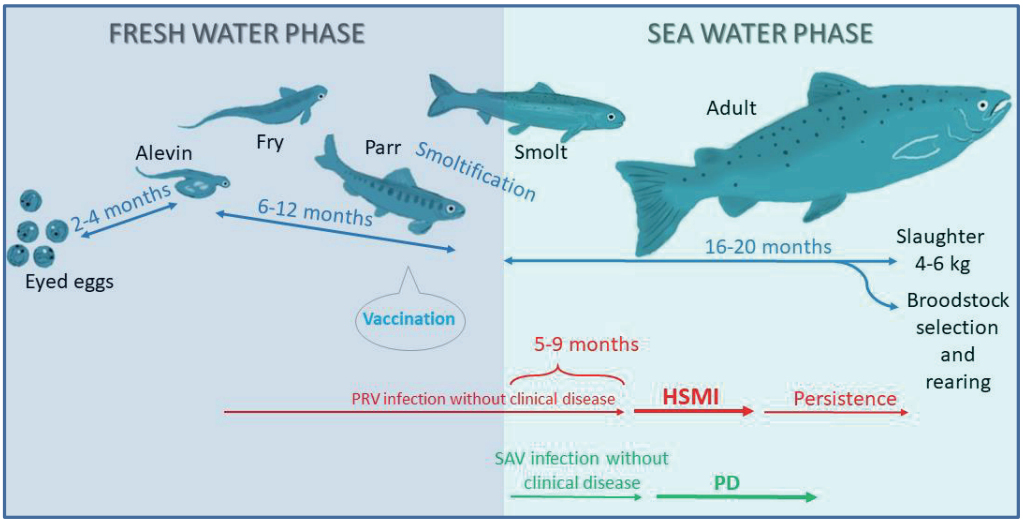

Figure 6. *Life cycle of farmed Atlantic salmon and common periods of PRV and SAV infection.*

Rearing fish at high densities under artificial and often stressful conditions imposes considerable risks of losses from outbreaks of infectious diseases and may have unforeseen adverse effects on wild fish populations by dissemination of pathogens. An overcrowded place with immunocompromised hosts

is an ideal environment for pathogens to spread and infect new hosts. RNA viruses mutate frequently, and for viruses with segmented genomes, reassortment of whole genomic segments may occur. More infected fish leading to more virus produced also means that mutations and reassortment will happen more often. The farming environment leads to an altered microbe evolutionary pressure and can increase the virulence of the infectious agent (Cressler *et al.*, 2016). The distance between fish populations are normally considerable in the wild, and there will be a selection for virus that give long-lasting infections with mild disease. In farming conditions however, the density of fish is much larger, giving opportunity for pathogens to rapidly infect new hosts. This will increase the selection for virus with rapid replication and spread, often correlated with higher virulence. In this way a virus that infects wild salmon without giving disease or only giving mild disease can change its characteristics and give rise to disease and mortality in farmed salmon. With close contact with wild fish, transport of live fish and global trade, the risk of spreading viral diseases is high, and we will doubtless see new emerging viral diseases in the future.

Table 1. *Features of PRV and SAV*

	PRV	**SAV**
Family	*Reoviridae*	*Togaviridae*
Genus	*Orthoreovirus*	*Alphavirus*
Genome	dsRNA 10 segments At least 10 ORFs 23 320 nucleotides	+ssRNA Not segmented 2 polyprotein ORFs 11,000-12,000 nucleotides
Proteins	At least 8 Structural and 3 non-structural	5 structural 4 non-structural
Capsid	Icosahedral double	Icosahedral single
Envelope	No envelope	Spherical
Size (diameter)	≈ 75 nm	≈ 60-70 nm
Subtypes	PRV-1-3	SAV-1-6

1.3.1 Piscine orthoreovirus

Piscine orthoreovirus (PRV) is a reovirus belonging to the genus *Orthoreovirus.* It is non-enveloped, has a double-layered icosahedral capsid and a segmented double-stranded RNA genome. The *Reoviridae* family name comes from Respiratory Enteric Orphan virus (Sabin, 1959), indicating in which tissues it was first found and that no disease was associated with it. Reoviruses have a wide

host range, including vertebrates, invertebrates, plants, and fungi (Urbano & Urbano, 1994). The family *Reoviridae* contains five genera that can infect mammals, birds and fish: *Orthoreovirus*, *Coltivirus*, *Rotavirus*, *Aquareovirus* and *Orbivirus*. Other genera infect plants and insects (Attoui *et al.*, 2011).

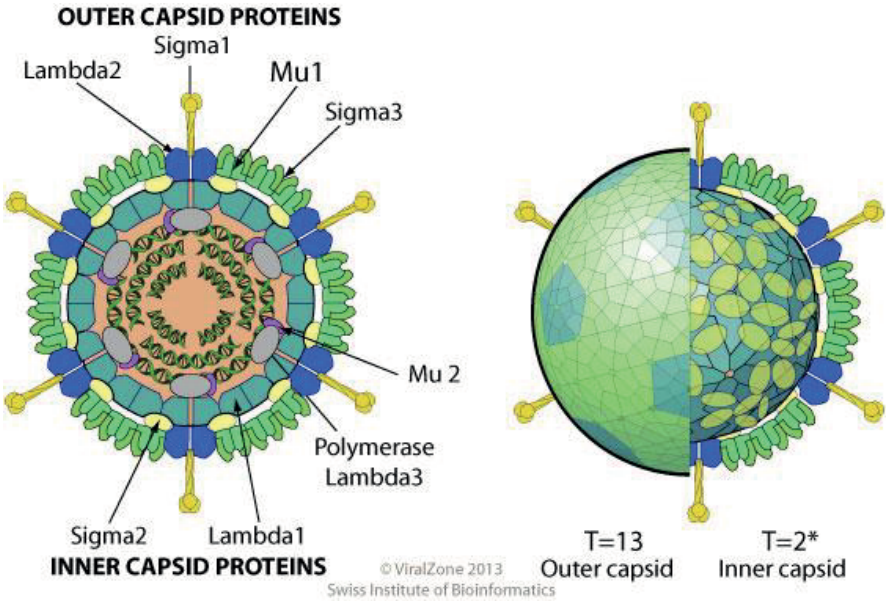

Figure 7. *Orthoreovirus proteins and structure. Source: ViralZone: www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics*

At least three subtypes of PRV, called PRV-1, PRV-2 and PRV-3, can cause disease in farmed salmonids. PRV-1 causes HSMI in Atlantic salmon (Palacios *et al.*, 2010, Wessel *et al.*, 2017) and jaundice syndrome in Chinook salmon (*Oncorhynchus tshawytscha*) (Di Cicco *et al.*, 2018). PRV-2 causes erythrocytic inclusion body syndrome (EIBS) in coho salmon (*Oncorhynchus kisutchi*) (Takano *et al.*, 2016). PRV-3 causes anemia and HSMI-like heart pathology in rainbow trout (*Oncorhynchus mykiss*) (Olsen *et al.*, 2015, Hauge *et al.*, 2017, Vendramin *et al.*, 2019, Adamek *et al.*, 2019). PRV-3 has also been suggested as the causative agent of proliferating darkening syndrome in brown trout (*Salmo trutta*) (Kuehn *et al.*, 2018), but this is disputed (Fux *et al.*, 2019). The PRV subtypes can also cross-infect between salmonid species.

PRV consist of at least eight structural and three non-structural proteins (Markussen *et al.*, 2013). The structural proteins of orthoreovirus are organized in an outer and an inner capsid (figure 7). The

outer capsid consists of μ1, σ1, σ3 and λ2 (Reinisch *et al.*, 2000). Spikes are formed by trimers of σ1. The inner capsid is made up of λ1 and σ2 proteins and small amounts of λ3 and μ2 (Reinisch et al., 2000). Studies concerning the functions of PRV proteins are limited (Wessel *et al.*, 2015a, Key *et al.*, 2013), but based on sequence homology, we can use the mammalian orthoreovirus (MRV) as a model for predicting functional properties of PRV-proteins. In MRV the σ1 protein attaches to the cell surface receptor junction adhesion molecule-A (JAM-A) and the virus enters the cell trough receptor mediated endocytosis (Guglielmi *et al.*, 2006). In the endosome, the outer capsid is proteolyzed, and an infectious subviral particle (ISVP) is left. The ISVP is further turned into a core particle that enters the cytoplasm (Smith *et al.*, 1969, Borsa *et al.*, 1973, Shatkin & LaFiandra, 1972). ISVPs can also be formed by extracellular proteolysis, for example in the intestine, and may enter the host cell using receptor-independent pathways (Schulz *et al.*, 2012). μ1 covers most of the ISVP surface and is proteolytically cleaved into μ1c and μ1n. μ1n is required for membrane penetration (Liemann *et al.*, 2002, Zhang *et al.*, 2006). Transcription occurs in the cytoplasm, but within the core particles. The plus-strand transcripts exit the core particle and are translated in the cytoplasm by host ribosomes. The assembly of viral proteins and genome takes place in the cytoplasm in so-called viral factories. The non-structural proteins are not part of the viral particle but are required for viral replication. μNS is responsible for the formation of viral factories (Haatveit *et al.*, 2016). Neither dsRNA nor negative-strand RNA is found free in the cytoplasm during viral replication (Tytell *et al.*, 1967). It is not known how PRV escapes the host cell. PRV-2 and PRV-3 infections are associated with anemia, and possible viral lysis of infected red blood cells. PRV-1 has not been associated with obvious anemia, but there might be mild anemia, or the loss of erythrocytes could be compensated by increased erythropoiesis. However, virus-induced lysis has not been shown. Orthoreoviruses are commonly released from infected cells through the disrupted cell membrane following cell death (apoptotic or non-apoptotic) (Fields *et al.*, 2007, Richardson-Burns & Tyler, 2004). Some orthoreoviruses can induce autophagy in infected cells (Niu *et al.*, 2019, Wu *et al.*, 2017, Tao *et al.*, 2019). Autophagy is a cellular function that can both inhibit and benefit virus replication. One way autophagy can be exploited by viruses is by non-lytic virion release (Dong & Levine, 2013). It can be speculated that PRV escapes infected cells in extracellular vesicles by this or other pathways.

PRV-1 is ubiquitous in Norwegian aquaculture, and HSMI is now one of the most prevalent diseases in farmed Atlantic salmon (Kongtorp *et al.*, 2004a, Palacios et al., 2010, Finstad *et al.*, 2014). The disease was first reported in Norway in 1999 (Kongtorp et al., 2004a). PRV was found to be the associated in 2010 (Palacios et al., 2010), and found to be the causative agent in 2017 (Wessel et al., 2017). HSMI is also reported from farmed salmon in other countries (Ferguson *et al.*, 2005, Godoy *et al.*, 2016). A diagnosis of HSMI is based on clinical signs and histopathological changes in heart and

skeletal muscle. The mortality in an outbreak varies from insignificant to 20 %, but all fish are usually infected (Kongtorp et al., 2004a). At the first acute stage of infection, PRV-1 infects red blood cells (Finstad et al., 2014). Up to 50% of erythrocytes can be infected, and this peak infection phase is the primary dissemination phase of the virus. Infection of myocytes follows (Finstad *et al.*, 2012), and this is associated with inflammation in the heart- and red skeletal muscles (Palacios et al., 2010, Mikalsen *et al.*, 2012, Wessel et al., 2017). This inflammation is characterized by an influx of CD8+ lymphocytes in the heart (Mikalsen et al., 2012). Histopathological findings include myocarditis, epicarditis and myocardial necrosis as well as inflammation and degradation of myocytes in skeletal muscle (Kongtorp *et al.*, 2004b). The heart can regenerate, but PRV causes a persistent infection in farmed Atlantic salmon (Haatveit et al., 2017, Garver *et al.*, 2016, Kongtorp *et al.*, 2006) that can also be associated with melanized focal changes in skeletal muscle (Bjorgen *et al.*, 2019). Macrophages are target cells for PRV and are associated with these melanized foci. During the persistent phase, PRV are found associated with erythroid progenitor cells, erythrocytes, macrophages, melano-macrophages and other un-characterized cells in the kidney (Malik *et al.*, 2019).

PRV-1 infects farmed salmonids at the North American Pacific coast, Iceland and the Faroe Islands with no or mild disease (Di Cicco *et al.*, 2017, Garver et al., 2016), and the virus has been found in samples from Norway predating the first observation of HSMI (Dhamotharan *et al.*, 2019). A reassortment event before 1997, or accumulation of mutations over time is a likely cause of increased virulence leading to disease. The changes are most likely associated with the presence of unique amino acids in one or more of the σ3, p13 and µ1 proteins (Dhamotharan et al., 2019).

1.3.2 Salmonid alphavirus

Salmonid alphavirus (SAV), (formally named *Salmon pancreas disease virus*), is a virus of the family *Togaviridae* and genus *Alphavirus*. It is a single-stranded positive-sense RNA virus causing pancreas disease (PD) in Atlantic salmon. The virus has an icosahedral capsid surrounded by an envelope (figure 8). Alphaviruses infect a range of different vertebrates. They are generally considered to be arthropod-borne viruses, but this is not the case for SAV. SAV has been classified into six subtypes, SAV-1-6, with distinct geographical distributions. The subtype classification is based on the nucleotide sequence of nsP3 and E2 (Fringuelli *et al.*, 2008), and only SAV6 is a different serotype

(Graham *et al.*, 2014). There is a good cross-neutralization of antibodies between all the other subtypes (Graham et al., 2014). SAV-2 and SAV-3 are the two subtypes found in Norway.

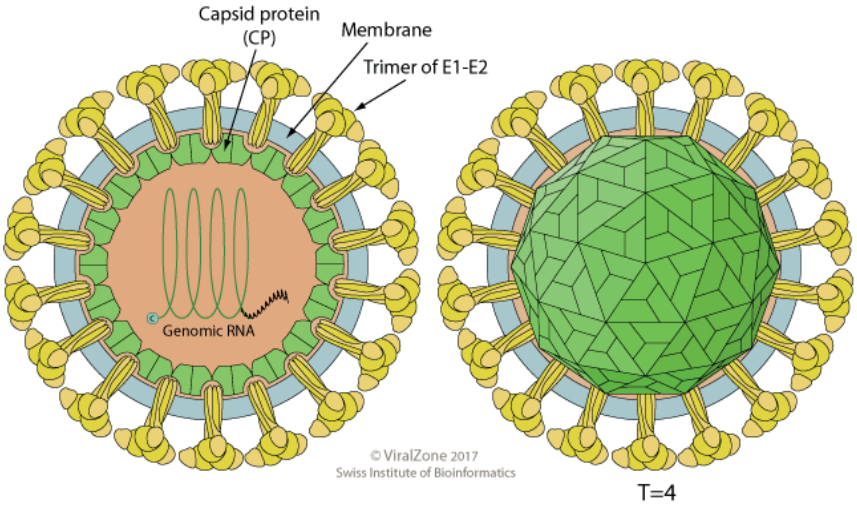

Figure 8. *Alphavirus proteins and structure. Source: ViralZone: www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics*

The SAV genomic RNA is around 12 kb long and consists of two open reading frames (ORFs). The first encodes the non-structural proteins (nsP1-4), and from the second, subgenomic RNA encoding the capsid proteins (Cap, E3, E2, 6k and E1), is made (Weston *et al.*, 2002). The structural proteins are expressed as a polyprotein, and then the proteins are cleaved off post-translationally. The capsid is made up of Cap proteins, and tetrameric heterodimers of the glycoproteins E1 and E2 form spikes through the envelope. The C-terminal cytoplasmic domain of E2 interacts with the capsid proteins. The non-structural proteins are responsible for replication and transcription of the viral genome. Replication takes place in the cytoplasm, and the genomic positive-strand RNA serves directly as messenger RNA for protein synthesis, and as the template for the generation of minus-stranded RNA (Strauss & Strauss, 1994). Replication starts with the E2 protein attaching to a cell surface receptor triggering endocytosis and formation of a cytoplasmic endosome containing the virus particle (Herath *et al.*, 2012b). Then the endosomal and viral membrane fuse and the nucleocapsid is released to the cytoplasm where replication occurs. E1 and E2 are glycosylated and translocated to the cell surface, the nucleocapsid is assembled and budding occurs as the nucleocapsid is wrapped in the E1E2-containing cell membrane (Hikke *et al.*, 2014, Jose *et al.*, 2009).

PD is endemic in Ireland, Scotland and two thirds of the Norwegian salmon growing area. Clinical signs are transient severely reduced appetite, lethargy, increased mortality, fecal casts and poor growth performance, with mortality rates up to 60 % during an outbreak (McLoughlin & Graham, 2007). The subtypes vary in pathogenicity, SAV2 infection has a lower mortality than SAV3 infection, and primarily results in low feed conversion and the development of "runt" fish. SAV can infect a wide variety of cells, but heart, skeletal muscle and pancreas are the organs mainly affected by PD (Andersen *et al.*, 2007). Muscle satellite cells have been reported as targets for SAV2 in rainbow trout (Biacchesi *et al.*, 2016). Histopathology show changes in pancreas, heart and skeletal muscles depending on the time after infection. Acute changes include pancreatic acinar cell necrosis and inflammation in the periacinar tissue of the exocrine pancreas and multifocal necrosis of cardiomyocytes. Later, in the chronic phase, both inflammation and fibrosis of skeletal muscles can be seen. Subclinical infection or mild disease, especially in SAV 2 infected farms and in PD vaccinated fish, is not uncommon. A farm can stay asymptomatically infected for several months before a critical viral load is reached and clinical disease becomes evident (Graham *et al.*, 2006, Jansen *et al.*, 2010, Stene *et al.*, 2014a). Stressful events like handling can also be the cause of an outbreak of PD in an asymptomatically infected population.

Horizontal transfer following water currents is considered the main means of spread of the virus (Viljugrein *et al.*, 2009, Stene *et al.*, 2014b). However, wellboats could be an even more important pathway of transmission (Amirpour Haredasht *et al.*, 2019). How long SAV particles remain infectious and how they are affected by temperature, currents and other environmental factors is not known.

PD should be suspected if one of the following criteria is met: clinical signs consistent with PD, histopathological lesions consistent with PD, specific antibodies against SAV, detection of virus or contact with suspected or confirmed PD cases. A confirmed case requires two independent positive tests according to the OIE (Office International des Epizooties) manual for diagnostic tests for Aquatic animals (OIE, 2019). In Norway, detection of viral RNA with RT-qPCR and histopathology are usually used (Jansen *et al.*, 2017).

1.3.3 Antiviral immune responses

The innate immune system plays a critical role in the first line of defense against virus (Kawai & Akira, 2006). The type I interferon (IFN-I) system is an essential part of this protection. Type I interferons are cytokines that are induced and secreted when a host cell recognizes viral nucleic acids via PRRs. Interferon in itself has no antiviral properties, but when a cell produces IFN, IFN will bind to IFN-

receptors that are present on most cells and through the JAK/STAT pathway induce production of a large repertoire of different antiviral proteins. There are more than 300 different interferon stimulated genes in mammals, and exposure to interferon triggers a complex transcriptional program (Garcia-Sastre, 2017). The adaptive immune response can also be stimulated by IFN-I. There are at least six different classes of IFN-I in Atlantic salmon (Robertsen, 2018). Viruses do not contain microbe-specific structures in the same way as bacteria and fungi, since they are made from host-derived components. Viral nucleic acids (i.e. long dsRNA or DNA without CpG methylations) are virus-specific components recognized by PRRs. TLR3, TLR22, RIG-I and melanoma differentiation-associated gene 5 (MDA5) are receptors that can bind dsRNA in fish (Strandskog *et al.*, 2008, Chen *et al.*, 2017).

Because they are nucleated, teleost erythrocytes can be infected by virus. Virus-infected erythrocytes can express anti-viral genes like ifn-α, Mx, protein kinase RNA-activated, viperin and interferon-stimulated gene 15 (Dahle et al., 2015, Haatveit et al., 2017). Uninfected erythrocytes are also able to produce anti-viral proteins and other proteins involved in immunity (Morera et al., 2011, Nombela & Ortega-Villaizan, 2018). As erythrocytes are the most numerous cell type in the bloodstream, small amounts of secreted molecules from each erythrocyte will be substantial when put together. Both genes involved in antigen presentation and PRRs like TLR3, TLR22, RIG-1 and dsRNA-activated protein kinase (PKR) is upregulated in PRV infected erythrocytes (Wessel *et al.*, 2018b, Dahle et al., 2015). These PRRs induce transcription of type I interferons through activation of interferon response factors (IRFs). Innate immune responses against SAV include upregulation of interferon and interferon-stimulated genes including Mx and viperin (virus inhibitory protein, endoplasmic reticulum-associated, interferon inducible) in both head kidney and heart (Johansen *et al.*, 2015, Herath *et al.*, 2012a). Several complement factors, including C3, C9, C1 and C4, are also up-regulated during SAV infection (Strandskog *et al.*, 2011, Braceland *et al.*, 2013), suggesting involvement of MAC (C9) in lysis of virus and of classic (antibody mediated) activation of complement (C4).

Viruses have developed several ways to evade the innate immune system. The main strategies are avoiding detection, inhibiting the protein cascade initiated by binding to the PRRs, inhibition of transcription of immune genes and inhibition of the immune cascade effector proteins (Workenhe *et al.*, 2010). During replication, the PRV dsRNA is protected in the core particle or within viral factories and is therefore not available to bind to TLRs in the endosome or RIG-1 and MDA5 in the cytosol. Processes like autophagy, cell lysis, misfolded viral proteins leading to unsuccessful replication or phagocytosis of infected cells could lead to exposed dsRNA. The MRV protein σ3 binds dsRNA and can thereby inhibit the innate response (Imani & Jacobs, 1988). PRV σ3 can also bind

dsRNA (Wessel et al., 2015a). Fish infected with the PRV-1 isolates that can cause HSMI seem to upregulate genes related to innate antiviral responses (Wessel et al., 2017, Wessel *et al.*, 2015b, Dahle et al., 2015) to a greater extent than isolates that do not induce HSMI (Garver et al., 2016). The ability to induce innate responses and subsequently humoral and cellular responses could therefore be related to virulence. The innate response persists for several weeks and can explain the findings that a PRV-1 infection seems to protect against a subsequent infection with SAV or IHN virus (Lund *et al.*, 2016, Vendramin *et al.*, 2018, Rosaeg *et al.*, 2017).

An effective immune response against viral infections usually depends on the activation of CD8+ T cells that can clear the infection by killing virus-infected cells. An efficient antiviral adaptive response is thought to be of the T_h1 type (at least in mammals, as not much is known about this in fish). The T_h1 cells are therefore required to skew the immune response in the right direction. HSMI pathology is mediated by CD8+ T lymphocytes recruited to the heart (Mikalsen et al., 2012). Gene expression studies indicate that T_h cells, macrophages and B cells are also present in hearts during HSMI (Johansen et al., 2015). Granzyme A is upregulated in heart, indicating a cytotoxic response and killing of infected myocytes. Other adaptive responses are an upregulation of both soluble and membrane-bound IgM in head kidney, and CD8 and granzyme in spleen (Johansen *et al.*, 2016).

While cytotoxic T cells are often involved in clearing an infection, antibodies, if present in advance, can neutralize virus and prevent infection. Antibodies can mediate a full protection against several viral diseases in mammals (Dorner & Radbruch, 2007). Antibodies can neutralize viral infectivity in a number of ways. Some antibodies, especially polymeric ones, can inhibit viruses by agglutination. They can also induce conformational changes in virus capsid or membrane resulting in non-infective virus particles. This has been shown for foot-and-mouth-disease virus (McCullough *et al.*, 1987) and Sindbis virus (Hernandez *et al.*, 2008). Enveloped viruses are neutralized by blocking of viral entry at different stages: attachment, fusion with cell membrane or fusion of the virus envelope to the vesicular membrane. Other viruses can be neutralized by blocking of the membrane penetration of either the whole virus, the viral core or the genome or intracellularly by facilitating binding to the tripartite motif-containing 21 (TRIM21), mediating ubiquination of the virus-antibody complex, targeting it for proteasomal degradation (Mallery *et al.*, 2010, Klasse, 2014). An enveloped virus can also be lysed by antibodies and complement by disruption of its membrane. Non-neutralizing antibodies are also produced after viral infection and bind specifically to virus particles without obstructing infectivity. They can however mediate antibody-dependent cellular toxicity and the killing of infected cells, the formation of a membrane attack complex and complement-mediated lysis and by Fc receptor-mediated phagocytosis. Non-neutralizing antibodies can also enhance infection by facilitating cell entry into phagocytic cells that lack a specific virus receptor. The

protective role of antibodies against viral infections in fish has been shown by passive immunization using serum or purified IgM from infected surviving rainbow trout (Olesen & Jørgensen, 1986, Lorenzen & Lapatra, 1999) and Pacific herring (Hershberger *et al.*, 2011). It cannot be ruled out that some of the protection is mediated by innate factors also present in serum. Neutralizing antibodies are detected in fish i.p. injected with SAV virus after around two weeks (McLoughlin *et al.*, 1996, Desvignes *et al.*, 2002). Serum taken from Atlantic salmon infected with pancreas disease up to 15 weeks earlier was shown to protect new fish from disease when injected around the same time as the fish were given an i.p. injection with pancreas disease infected kidney homogenate (Houghton & Ellis, 1996). Fish surviving a PD outbreak are less susceptible to SAV infection, suggesting that a protective immune response is present. Passive immunization of fish also indicate that these antibodies are protective (Biering et al., 2005, Houghton, 1994). It is not known if PRV-infected fish produce neutralizing antibodies.

Behavioral fever, where the fish seek out water with higher temperatures has been observed as a response to infection in several fish species, including Atlantic salmon (Boltana *et al.*, 2018), to Poly I:C (synthetic dsRNA) in zebrafish (Rey *et al.*, 2017) and to LPS in rainbow trout (Grans *et al.*, 2012). An increase in temperature has been shown to increase viral clearance and modify the immune response in fish (Avunje *et al.*, 2012, Boltana *et al.*, 2013, Boltana et al., 2018). Potential behavioral fever induced by PRV or SAV infections has not been studied.

1.3.4 Vaccination against viral diseases in salmon

Vaccination is an effective way of preventing infections. The concept of vaccinology depends on the mechanism that a previous encounter with a pathogen will give a faster and "stronger" response the second time the same pathogen is faced. For this to happen the host must possess some kind of immunologic memory.

The principle of vaccination has been exploited for centuries. In 10th century China, powdered smallpox scabs from mild smallpox cases were administered intranasal to give a milder version of smallpox than would occur by natural infection. A variant with inoculation was used in Turkey and was introduced to England by Lady Mary Wortley Montagu in 1722. The methods generally gave a milder variant of smallpox and a life-long protection against the disease, but still up to one in 50 recipients died as a result of the procedure. The procedure was still considered worth the risks as a smallpox epidemic usually killed 10-20 % of the population. In 1774, Dorset farmer Benjamin Jesty infected his family with cowpox to protect them during a smallpox epidemic (Pead, 2003). Others likely used the procedure as well, since it was well known that people who had been infected with

cowpox never got smallpox. Over 20 years later, in 1796, Edward Jenner was the first to apply scientific principles to the subject as he used the procedure on several patients (Jenner, 1799). Today, we routinely vaccinate children and adults, farmed animals, pets and wild animals against a number of viral diseases. Due to vaccinations, smallpox (caused by the variola virus) was declared eradicated in 1980. Rinderpest is a disease caused by a *morbillivirus* that affected cattle. Because of vaccination, the disease was declared eradicated in 2011, as the second infectious disease ever.

There are four main types of vaccines, inactivated vaccines, attenuated vaccines, subunit vaccines and DNA vaccines (figure 9). Subunit and inactivated vaccines mainly elicit a humoral response with formation of antibodies and are generally effective against extracellular pathogens. Antibodies can also stop intracellular pathogens from infecting cells. They are safe to use, but often require booster vaccinations. Attenuated vaccines give a controlled sub-clinical infection that more closely imitate the natural infection and are in general better at eliciting protective long-lasting immunity, mainly in relation to T cell-mediated and mucosal responses. DNA vaccines also have the potential to induce both cellular and humoral immune responses due to intracellular protein expression.

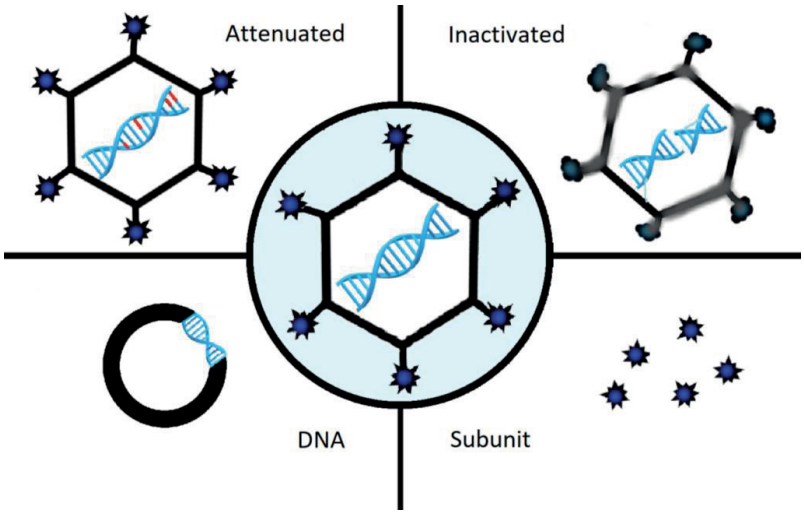

Figure 9. *Illustration of four main vaccine types with original virus in the middle. An attenuated virus is a laboratory-weakened version of the original virus that is less virulent or less able to replicate in a host. Inactivated vaccines are whole virus particles that have been destroyed with chemicals, heat or radiation. In DNA vaccines, parts of the virus genome are taken up by host cells that then produce the encoded protein and display it on MHC on their surface. Subunit vaccines include only some of the virus proteins.*

Most vaccines contain an adjuvant to enhance the immune response. Many types of adjuvant exist, and many of the mechanisms they work by are not fully understood. They can include a danger signal, a non-self microbial signal, facilitate antigen delivery to secondary lymphoid organs or provide a depot effect (Singh & O'Hagan, 2003). The most common effect is through triggering the local innate immune response.

The earliest publication on fish vaccinations was in 1942 when Duff (Duff, 1942) worked on oral vaccination of cutthroat trout (*Oncorhynchus clarkii*) against *Aeromonas salmonicida*. Today farmed Atlantic salmon are vaccinated against several diseases. Most vaccines used for fish are inactivated whole bacteria/virus with an oil-based adjuvance injected i.p. (Sommerset et al., 2005, Brudeseth *et al.*, 2013) The oil also functions as a depot. In the early days of salmon farming in Norway, bacterial diseases were the biggest challenge. 50 metric tons of antibiotics were used yearly at the most. Since the first oil-based vaccine was launched in 1992 against furunculosis in Atlantic salmon, oil-adjuvant vaccines have been effective at controlling bacterial diseases in salmon farming (Lillehaug *et al.*, 2003). Vaccines against bacterial infections have contributed to an extreme reduction of antibiotics use in aquaculture. Now less that one metric ton of antibiotics are used yearly. At the same time production has increased from less than 100 000 to more than 1 000 000 tons. This would not have been possible without vaccines. Viral diseases are now considered one of the biggest challenges facing the industry. Commercial vaccines based on inactivated virus (SAV and infectious pancreas necrosis virus (IPNV)) or recombinant virus protein (IPNV) are in use, but the protection is suboptimal (Evensen & Leong, 2013). The vaccines are oil-based and administered intraperitoneally. Other routes for administration that can be used in fish are oral, immersion or intramuscular, each with their own advantages, challenges and limitations. An inactivated PD vaccine based on SAV1 has been available in Norway for over a decade but has not decreased the number of PD outbreaks. It has, however, been shown to reduce the severity of the disease and to reduce viral shedding (Bang Jensen *et al.*, 2012, Skjold *et al.*, 2016). A new inactivated PD vaccine and a DNA vaccine, both based on SAV3, have recently become available. The effects of these vaccines in the field remain to be seen.

Some claim to find a correlation between antibody titers in ELISAs or neutralization assays and the protection acquired after a vaccine or challenge trial, including studies on IPNV (Frost & Ness, 1997, Frost *et al.*, 1998, Munang'andu *et al.*, 2013). An inactivated infectious salmon anemia virus (ISAV) vaccine showed a correlation between antigen dose, level of specific antibodies and protection (Lauscher *et al.*, 2011) and a DNA vaccine against ISAV with interferon as adjuvant has been shown to induce ISAV-specific IgM (Chang *et al.*, 2015, Caruffo *et al.*, 2016). A replicon (self-amplifying RNA encoding both the gene of interest and all elements necessary for replication) vaccine has also shown protection against ISAV when administered intramuscularly, but the antibody levels were low and

did not correlate with protection (Wolf *et al.*, 2013, Wolf *et al.*, 2014). Others have also found that the mechanism of protection is independent of the antibody level (Lillehaug, 1997). According to Jørgensen et al., the survival from ISAV-infection was correlating more strongly to the level of cell-mediated responses than to the level of humoral responses, based on gene expression analysis (Jorgensen *et al.*, 2008).

In evaluation of mammalian vaccines, antibody levels are the most widely used correlate of protection (Plotkin, 2010). Generally, vaccine development in fish has been empirically-based and has not always been done as in mammals where there is a good understanding of the protective mechanisms and defined correlates of protection before the vaccines are licensed (Rappuoli & Aderem, 2011). Relative percent survival rates are often used as the only measurement of effect in fish vaccines. Whether the protection is mediated through innate, cellular or humoral responses and if these responses block viral entry, prevent pathogen dissemination or prevent tissue damage is usually not known.

It is not clear what constitutes a protective immune response against PRV or SAV infections. There has been found neutralizing antibodies in Atlantic salmon immunized with several different SAV-vaccines, for example an inactivated vaccine (Xu *et al.*, 2012) and a DNA vaccine encoding the structural SAV polyprotein (Chang *et al.*, 2017). An inactivated PRV vaccine made from purified virus particles was shown to mediate protection against HSMI when vaccinated fish were i.p injected with PRV, but not to the same degree when fish were infected by cohabitation (Wessel *et al.*, 2018a). One reason for this, suggested by the authors, is that an i.p. injected vaccine fails at eliciting protection at mucosal surfaces. This is a very likely reason, as systemic vaccines often produce systemic protection, whereas mucosal vaccines can elicit both mucosal and systemic protection as seen in mice (Belyakov *et al.*, 1998) and perhaps in salmon (Mutoloki *et al.*, 2015). To make an inactivated or attenuated vaccine in large scale, it is necessary to cultivate the pathogen. As there is no cell line available for cultivation of PRV, other vaccine types must be used. A DNA vaccine containing the non-structural proteins of PRV has been shown to induce a moderate protection against HSMI (Haatveit *et al.*, 2018). No significant upregulation of the IgM genes was seen, but this was measured six weeks after vaccination, which could be too early for antibodies to develop.

The lack of an immune correlate of protection hampers vaccine development. When testing vaccine efficacy, both survival, virus level in the fish and serology can be used. A high level of antibodies correlating with protection does not prove that antibodies are responsible for the protection but can still predict protection through a correlation with other protective immune responses.

1.4 SEROLOGY, IMMUNOASSAYS AND ANTIGEN-ANTIBODY BINDINGS

The word serology comes from the study of serum. Now the term is most often used for the detection of antibodies in serum or other bodily fluids. Serology relies on an appropriate immunological response in the host that is diagnostically detectable. In contrast to in mammals, serology has not been widely used in salmonids. The interest in the salmonid immune system has advanced in parallel with the development of modern aquaculture, decades after the study of the mammalian immune system started. For diagnostic purposes, histology and PCR have been the dominating tools as they were already available. Another factor is that the immunoglobulin isotype most common in fish, IgM, has a lower affinity and is more cross-reactive than the mammalian IgG. As fish IgM in general is of lower specificity than mammalian IgG, there is an increased risk of detecting low affinity cross-reactive antibodies in non-infected fish and hence, to have false positive results. The term antibody titer is often used in serology and is defined as the highest dilution of serum that still results in a detectable antibody binding. The presence of antibodies in serum is not evidence for active or persistent infection, as antibodies can be present for a longer period after the pathogen is eliminated. This is a limitation for the diagnostic use of serology. At the same time, it can be an advantage when it is used epidemiologically to identify individuals or populations that have been in previous contact with a pathogen, when pathogen levels are too low to be detected with PCR or when pathogens are present in tissues not submitted for PCR testing. In mammals, serology is widely used for diagnostic purposes as well as in research, in surveillance programs to prove absence or prevalence of infection, to confirm suspected cases of infection and to certify animals for export or import.

An immunoassay is an analytical technique using either antibodies to detect a molecule, or an antigen to detect a specific antibody in a sample. The sensitivity and specificity depend on the antibody affinity and the specificity of the antibody-antigen binding. The first immunoassay was developed by Rosalyn Yalow and Solomon Berson in 1959-1960 (Yalow & Berson, 1959), used radioactive labeling for detection and was used to measure insulin levels in blood. Ten years later, the enzyme-linked immunosorbent assay (ELISA) was developed (Engvall & Perlmann, 1971, Van Weemen & Schuurs, 1971). ELISAs are still widely used today and use an enzymatic reaction for detection. The enzyme is coupled to an antibody, and often catalyzes a reaction that produces an observable color-change in the presence of the target molecule. ELISAs are relatively low-cost analyses that are easy to perform. They are, in contrast to multiplex bead assays only able to detect the level of a single analyte in each assay and require a higher sample volume and more antigen per analyte measured. ELISAs with whole viral particles or recombinant viral proteins as capture antigen and virus neutralization assays have been used in diagnostics in aquaculture (Munang'andu et al.,

2013, Kim *et al.*, 2015, Kibenge *et al.*, 2002, Mikalsen *et al.*, 2005, Rocha *et al.*, 2002), but serology in general has not been commonly used for diagnosis and surveillance purposes. A serological immunoassay is a quantitative analysis based on the reaction between an antigen and an antibody to measure the concentration of the antibody in a sample. It is often used to detect antibodies towards a pathogen in a sample using parts of the pathogen as antigen. ELISAs can be direct (serological), indirect (serological), sandwich (measuring amount of antigen with capture antibodies) or competitive (direct, indirect or sandwich, but using an inhibitor antigen/antibody to compete with the antigen/antibody of interest).

1.4.1 Bead-based multiplex immunoassay

The main part of this work has been done using Luminex' xMAP (x = analyte, MAP = Multi-Analyte Profiling) technology (Kettman *et al.*, 1998). This technology has been successfully used to detect mammalian antibodies for more than a decade (Powell *et al.*, 2013, Guigue *et al.*, 2014, Koffi *et al.*, 2015). Microscopic (6,5 µm) magnetic beads (MagPlex microspheres) are used, and up to 100 different analytes can be measured simultaneously in one sample. An antigen (or antibody) is bound covalently to the bead surface and just as in an ELISA, both indirect/serological, capture sandwich and competitive formats can be used for detection.

xMAP has all the advantages that ELISAs has, but direct fluorescence is used for detection without the requirement of enzyme activity. Each bead set is uniquely coded using different concentrations of two fluorescent dyes, and fluorescence from phycoerythrin (PE) is used as a reporter for bound antibodies. There are also beads available containing three fluorescent dyes, making it possible to analyze up to 500 different analytes simultaneously. The beads are read using the principles of flow cytometry, which is often used to differentiate cells based on size, granularity, and complexity. The flow cytometer was invented in 1968 (Dittrich & Gohde, 1969), and ten years later, beads of different sizes was proposed for flow cytometry analysis to detect analytes in samples (Horan & Wheeless, 1977). In 1997, color coded separation was introduced (Fulton *et al.*, 1997). This could be expanded to more beads than size coding, and is now part of the xMAP technology commercialized by Luminex corporation (Kettman et al., 1998).

The beads consist of a polystyrene core, magnetite, two fluorescent dyes and surface carboxyl groups. The magnetite makes the beads paramagnetic, meaning that they respond to magnetic fields, but are not individually magnetic. They are therefore attracted to a magnetic field but will be in suspension in the absence of a magnetic field. This facilitates easy separation of beads from

solution during washing steps. The fluorescent dyes give each bead number a unique spectral signature for identification. The desired proteins can be covalently bound to the carboxyl groups on the surface of the microspheres. First the carboxyl groups on the microspheres are “activated” by 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) reagent in the presence of N-hydroxysulfosuccinimide (S-NHS). This forms a reactive S-NHS-ester intermediate that reacts and forms a covalent amide bond with primary amines on the protein. Primary amines are found either on the N-terminal of the peptide chain or in lysine side chains. The proteins will typically be coupled in random orientation as they can have many lysine side chains available for coupling.

Beads are first washed, before incubation with samples in 96 well plates. Then one or more incubation steps with antibodies and washing steps between them follow. The washing is done by using a magnet to keep the beads in place and throwing off the supernatant. The last antibody used is biotinylated and in the final incubation streptavidin-phycoerythrin (SA-PE) is added. After a final washing step, the beads are read using the Luminex 200 instrument. The fluid with the beads is drawn into an array reader, and the beads are read one-by one as they are passed through a flow cell. Two lasers excite each bead individually, and the emission signals from each bead is registered. The first laser excites the two identification dyes. The dyes are excited by the same wave length but emit different wave lengths. The emission is translated to the bead set identity. The second laser excites PE, and the fluorescence is registered (Houser, 2012). Typically, 50-100 beads of each bead set are read, and the result is given as median fluorescent intensity (MFI). Based on experiments using PE standards, the approximate molecules of PE per MFI for Luminex 200 is 23 PE/MFI at standard (low) PMT and 5 PE/MFI at high PMT.

When beads are incubated with the sample in solution, it results in more surface area exposed to the sample in three dimensions than traditional assays like ELISAs where antigens are adsorbed onto two-dimensional surfaces. As each bead set can be conjugated with different antigens, the assay can measure antibodies for several antigens at the same time in the same sample. When compared with ELISAs, the bead-based immunoassays have an increased dynamic range, are labor and time saving, give lower background, require smaller amounts of antigen for coating and of sample material, have a higher sensitivity and have a similar specificity (Powell et al., 2013). The technology can be used on plasma, serum, blood, urine, other bodily fluids, culture media and also on dried blood spots on filter paper. Other applications of the serologic bead-based assays are epitope mapping (Costa *et al.*, 2007) and analysis of protein functions and interactions with other proteins with enzyme assays and receptor-ligand assays. If antibodies are coupled to the beads, competitive immunoassays and capture sandwich immunoassays are possible. This can be used for proteomics studies (Schwenk *et al.*, 2010) or pathogen detection (Hoare *et al.*, 2016). If DNA sequences instead of proteins are

coupled to beads, like in the commercial QuantiGene Plex Gene Expression assay, gene expression analyses can be performed.

1.4.2 Antibody – antigen binding

The antibody-antigen binding is a reversible chemical interaction. The forces keeping the antibody and the antigen together is not strong covalent bonds, but weaker bindings like hydrogen bonds, van der Waals interactions, electrostatic bonds and hydrophobic forces. Water molecules in the binding interface stabilize the binding by filling "empty" spaces and forming hydrogen bonds (Braden *et al.*, 1995). The strength of these molecular interactions determines the affinity of the antibody towards the antigen. The area where the paratope of the antibody binds the epitope is comprised of a few amino acids, and is between 0,4 and 8 nm^2 in size (Van Oss, 1995). Because each single binding interaction only lasts for a fraction of a second, several weak bonds must be present simultaneously for the antibody-antigen binding to be stable. This requires steric complementarity between the epitope and the paratope (Reverberi & Reverberi, 2007), partly because van der Waals forces are extremely short-ranged. Antibodies can bind to all parts of an antigen, but in general parts with certain stereochemical properties, like a convex surface, negative electrostatic potential and amino acids containing certain side chains, have a higher antigenicity (Geysen *et al.*, 1987, Mian *et al.*, 1991, Davies & Cohen, 1996). When animals are immunized with the same antigen, most, but not all, show reactivity towards the same highly immunogenic parts (Getzoff *et al.*, 1987).

As all reversible chemical reactions, the antigen-antibody reaction $[Ab] + [Ag] \rightleftharpoons [AbAg]$ will go in both directions and will eventually reach equilibrium. At equilibrium, according to the law of mass action, the ratio between the left and right side of the equation will be constant and is called the equilibrium constant (K_{eq}). K_{eq} is also equal to the ratio between the association (k_a) and the dissociation (k_d) rate constant.

$$\frac{[AbAg]}{[Ab][Ag]} = \frac{k_a}{k_d} = K_{eq}$$

The greater the strength of the bond, the higher is the k_a and K_{eq}, and the more of the antibodies and antigens will be bound together in complexes. A high affinity antibody-antigen binding will have a high K_{eq}. If the concentration of the reactants changes, the K_{eq} is not affected, but the equilibrium will shift to minimize the effect of that change. This means that if we increase the amount of antigen, we will get more antigen-antibody complexes, and decreasing the antigen or antibody concentration, or both will decrease the amount of complexes. Other factors like temperature, pH and the ion

concentration in the solution can affect the K_{eq}. Within normal temperatures for antibodies, the change in equilibrium constant is minimal. Temperature is still important as the time it takes a chemical reaction to reach equilibrium varies significantly depending on the temperature. Optimal duration of incubation in antibody assays is therefore temperature dependent.

In reality, antibody-antigen interactions in an immunoassay are more complicated than the simple equation. Antigen recognition by antibodies depend on the antigen conformation and presentation on the beads. The antibodies in plasma are polyclonal antibodies, meaning that they are mixtures of antibodies with different affinities. Complex antigens can display several epitopes that each can be recognized by antibodies with different affinities, isotypes and glycoforms. It further complicates the antibody-antigen interactions that each antibody monomer has two binding sites. Teleost IgM is a tetramer and therefor contain eight binding sites. Avidity is the term used to describe the accumulated strength of multiple non-covalent binding interactions and is the functional affinity of an antibody molecule. Multiple bindings require the epitopes to be sufficiently close together and a flexibility of the antibody arms.

The ambient analyte theory is based on the law of mass action (Ekins, 1989). Microarray technologies are built on this theory (Ekins *et al.*, 1990). It states that when using microscopic spots, in contrast to macrospots (like ELISAs in 96 well plates), with a high concentration of capture molecules, the results are independent of both the concentration of the capture molecule and on the sample volume (figure 10). The small spots of capture molecule will bind target molecules locally without interfering with the overall concentration and chemical equilibrium in the sample. Even in the case of low target concentrations, the concentration of the target molecule does not change significantly. It has been described as sampling the environment without affecting it just as a thermometer measuring its surrounding temperature. If the amount of antibody captured by the capture molecules is insignificant compared to the total amount of antibody, the ambient analyte conditions are fulfilled. The thermometer analogue would be that the thermometer submerged in liquid absorbs heat until it has the same temperature as the liquid. The temperature measured does not differ from the original temperature of the liquid if the heat absorbed is an insignificant fraction of the total heat in the system. The measured temperature is independent of both the size of the thermometer and the volume of liquid if the thermometer is small enough compared to the volume of liquid (Wild, 2013). xMAP beads are three dimensional microspots and bead-based immunoassays fulfill the criteria of the ambient analyte theory (Poetz *et al.*, 2010).

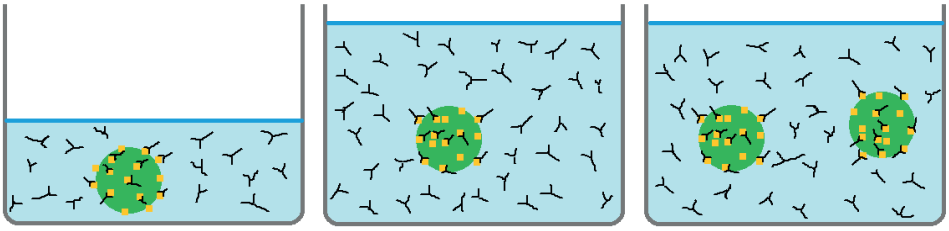

Figure 10. *The fractional occupancy of antigen binding sites by antibodies (66% in this figure) is independent of both sample volume and antigen concentration (number/size of beads and/or density of antigens on beads), but dependent solely on the antibody concentration in the medium provided that the antigen concentration (determined by size and number of beads, and the density of antigen on beads) is small enough.*

Sensitivity of an immunoassay is related to the limit of detection (Ekins & Edwards, 1997) and can be defined as the ability of the assay to detect small quantities of a target protein. In practice, this means how low amount of the protein can give rise to a signal above the background signal, or how small amounts of protein will give rise to an increase in the signal.

Strictly speaking, selectivity should be used instead of the often used "specificity" when describing an analytical method according to the International Union of Pure and Applied Chemistry (Vessman *et al.*, 2001), as specificity is an absolute term describing the ultimate of selectivity. Selectivity of an immunoassay then refers to the ability of the assay to detect a target molecule among other molecules that can be present in much higher concentrations.

1.4.3 Antigens and expression systems

Antigens are an essential part of an immunoassay. Both the sensitivity and the selectivity of an immunoassay are highly dependent on the antigen used and the antibodies' affinity towards that antigen. The antigen used needs to be similar enough to the native form for naturally occurring antibodies to recognize it.

Antigens are usually recombinant proteins, meaning that they are made by cloning the target gene into a host cell before making the host cell produce it. Production of proteins for this use can be done using recombinant cells from animals, bacteria, fungi or plants (Ma *et al.*, 2005, Ma *et al.*, 2003). It is possible to make proteins in vitro, using cell lysate instead of live cells. The lysate contains all the cellular components needed for protein synthesis (tRNA, ribosomes, amino acids, initiation,

elongation and termination factors), and the selected RNA sequence can be added. In vitro reactions have limitations, and often fail at producing correctly folded proteins. Proteins are often cleaved and modified as a normal process of expression, and this is not possible using in vitro reactions. The most commonly used expression system is the bacteria *E.coli* (Baneyx, 1999), and despite some limitations, a majority of antigens could be made in this expression system (Structural Genomics *et al.*, 2008). Each expression system has its advantages and challenges. Cost, production time, production capacity, product quality, temperature requirements, contamination risk and post-translational modifications are important when choosing expression systems. Stability, solubility and toxicity to the host cell, as well as the impact of having proteins from the host cells mixed in with the recombinant protein must also be considered. One must also decide if the whole protein should be expressed, or just a part of it, what vector and cloning strategy to use and how to purify the protein once it is produced.

Proteins made in common prokaryotic expression systems like *E. coli* are limited by the lack of eukaryotic posttranslational modifications like glycosylation, phosphorylation and acetylation that can be the key to correct protein folding, function and recognition by antibodies. Conformational epitopes are lost without correct folding. In eukaryotic cells, up to 98% proteins are N-terminally acetylated (Johnson *et al.*, 2010).The folding of the proteins overexpressed in prokaryotic cells can be incorrect, particularly if the proteins must be solubilized from inclusion bodies. Lipid modification can facilitate easier purification of antigen and might affect the folding and presentation of certain protein domains. It is a common post-translational process in bacteria. Through the sec or Twin arginine translocase (Tat) pathway lipoproteins are transported from the cytoplasm, through the inner membrane to the periplasm. The lipoproteins have a so-called lipobox, [LVI][ASTVI][GAS]C, at the N-terminal, and through three consecutive enzymatic steps the cysteine is fatty acetylated. This makes the trans-membrane part in the otherwise hydrophilic part of the protein. A “S/T-R-R-F-L” signal sequence directs the lipoprotein to Tat. Tat is required even for the earlier lipid modification to take place (Shruthi *et al.*, 2010).

Advantages of using eukaryotic expression systems involve correct post-translational modifications, protein folding, protein processing, disulfide bond formation and glycosylation. A eukaryotic system suitable for producing high levels of recombinant proteins is the baculo/insect cell expression system The sf9 cells used in this system is derived from pupal ovarian tissue of the lepidopteran (moth) species Fall armyworm (*Spodoptera frugiperda).* Correct protein folding and post-translational modifications are possible (Kost *et al.*, 2005). Baculoviruses are double-stranded DNA viruses. They can infect and kill many invertebrate species, including insects (Harrap, 1972). *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is the most commonly used baculovirus for

protein expression. A recombinant baculovirus DNA, where the polyhedron gene is exchanged with the gene(s) of interest, is used to transfect sf9 cells, leading to production of both protein and recombinant infectious virus. Because the expression of the polyhedrin gene is controlled by a strong polyhedrin promotor, insect cells infected or transfected with the recombinant baculovirus can overexpress target proteins with very high yields. The production of protein from the transfected cells should be evaluated, and if found satisfactory, the virus produced by the transfected cells is used to infect more cells. These cells will have a higher protein yield than the transfected cells and produce more virus.

Although insect cells are eukaryotic, some post-translational pathways are different than in higher eukaryotes. One example is glycosylation, where a similar, but different pathway leads to different modifications (Kost et al., 2005). The final step of glycosylation, addition of sialic acid to the glycan is also lacking in insect cells (Marchal *et al.*, 2001). No glycosylation is found in orthoreoviruses (Attoui et al., 2011). The E1 and E2 proteins of SAV are glycoproteins (Hikke et al., 2014). Both *E. coli* and sf9 cells are kept at a higher temperature than what is considered ideal for PRV and SAV, *E. coli* at around 37 °C and sf9 cells around 27°C.

Cell lines from fish can be used to produce a correctly folded and modified antigen or be infected with virus and produce virus particles. Still the folding of the protein can be temperature dependent as is the case for E2 in SAV. Because of this, the formation of SAV particles is dependent on lower temperatures (Hikke et al., 2014, Metz *et al.*, 2011). SAV E2 also require co-expression with E1 for correct translocation and presentation on the cell surface. Many cell lines, for example chinook salmon embryo (CHSE) cells are capable of propagating SAV, however no cell line is available for PRV propagation. To find fish cell lines that can grow fast and produce high amounts of protein is challenging.

Even if the modifications and the folding in the expression system is satisfactory, the protein needs to be isolated and purified. The use of purification tags is common (Amarasinghe & Jin, 2015). The resulting fusion protein can be more stable or more soluble in the host cell than the native protein and it may be a convenient "tag" for detection or purification. Sometimes the tag can also affect production yield and folding of the fusion protein. The tag can later be cut off using a sequence specific protease. Commonly used tags are the His-tag (6-10 consecutive histidine residues) and GST-tag (the enzyme glutathione S-transferase), where the gene of interest is cloned as a fusion protein with the tag on the amino or carboxyl end.

1.5 KNOWLEDGE GAPS

In general, the mechanisms that regulate B and T cell activation and function in Atlantic salmon are incompletely understood, as immunological research in salmon has lacked tools, including good monoclonal antibodies against cell markers, used to describe key elements involved in immunological protection.

PRV and SAV are important viruses in Norwegian aquaculture. Parallel to this work, the causal relationship between PRV and HSMI was proven (Wessel et al., 2017), and two new subtypes of PRV were discovered. An inactivated PRV vaccine was also proven to partially protect against HSMI (Wessel et al., 2018a). Despite this, knowledge about the pathogenesis and possible protective immune mechanisms, including the role of antibodies, is still lacking. Viral infections can be controlled by vaccination. However, to make good vaccines for Atlantic salmon, a better understanding of both the pathogen, the salmon immune system and the interaction between them is required. The identification of targets for antibodies on PRV or SAV could be useful for development of sub-unit vaccines and antigens that can be used in immunodiagnostics. Specific antibodies, especially neutralizing antibodies, can correlate with long-term protection against some viruses, and simple, fast, sensitive and specific ways of measuring them are highly warranted. When this PhD project was initiated, there were no described methods to detect antibodies against PRV. It was not known if specific antibodies were produced and, if so, whether they were neutralizing or not. Neutralizing antibodies are produced in SAV-infected fish and used in diagnostics, but virus neutralization assays are time-consuming. The xMAP assay is a promising method for easy antibody detection but had not yet been used for this purpose in fish when this project started. The use of this method could be complicated by polyreactive antibodies, since fish IgM has lower specificity than mammalian IgG, and since the highest affinity antibodies made in fish most likely have a much lower affinity compared to mammalian antibodies.

2 AIMS OF THE STUDY

Main objective

The main objective of this work was to study the immune responses during viral infections in Atlantic salmon, with focus on virus-specific antibodies.

Sub goals:

1. Establish bead-based immunoassays to detect salmon antibodies against *Piscine orthoreovirus* and Salmonid alphavirus.
2. Study the antibody production and dynamics and the cellular immune responses in Atlantic salmon during viral infections.
3. Optimize the bead-based antibody detection assay by using different strategies for antigen production to reduce cross-reactivity and unspecific binding.

3 SUMMARY OF PAPERS

Paper 1

A bead based multiplex immunoassay detects *Piscine orthoreovirus* specific antibodies in Atlantic salmon (Salmo salar)

In this paper, the goal was to use the bead-based immunoassay to detect antibodies against PRV in infected Atlantic salmon. The PRV structural proteins µ1c, σ1 and σ3 were made recombinant in *E. coli* and the non-structural protein µNS was made in the baculo/insect cell system, followed by coating on microscopic magnetic beads. IgM binding to µ1c and µNS was detected in plasma from PRV-infected fish from approximately two weeks after the peak phase of PRV infection, in a cohabitation challenge trial that lasted for 10 weeks. The antibody production increased throughout the infection trial, while the typical HSMI pathology became gradually less prominent. This was the first detection of antibodies against PRV and the first use of this immunoassay to detect IgM in Atlantic salmon.

Paper 2

Detection of Salmonid IgM specific to the *Piscine orthoreovirus* outer capsid spike protein sigma 1 using lipid-modified antigens in a bead-based antibody detection assay

The aim of paper II was to optimize the immunoassay used in paper I to detect antibodies against the PRV spike protein σ1, which is a putative receptor-binding protein and neutralization target. We tested σ1 on the beads in paper I but did not detect antibodies against it. The recombinant σ1 protein used in this paper was made with a lipid modification as a means to improve antigen folding. With this modified antigen, we detected significant increase in σ1 specific IgM antibodies in plasma of PRV infected Atlantic salmon from week 10 post challenge in a cohabitation challenge. An unspecific IgM background binding was observed in PRV negative fish and to non-PRV proteins used as controls in infected and control fish. Heat-treatment of plasma was shown to decrease this background binding. Pre-adsorption of plasma against antigens indicated that the antibodies binding to σ1 were specific. As this protein is expected to be the receptor-binding protein of PRV, antibodies against it could be neutralizing. This shows that a PRV infection increases the levels of both specific PRV-and non-specific antibodies in salmon plasma. The assay was also used to detect anti-σ1 antibodies in PRV3-infected rainbow trout, both in a challenge experiment and in a field outbreak.

Paper 3

IgM directed against Salmonid alphavirus antigens can be detected after a natural pancreas disease outbreak, using a bead-based immunoassay

In paper 3, we aimed at detecting antibodies against Salmonid alphavirus (SAV) in Atlantic salmon. Infected fish are known to produce neutralizing antibodies against the virus and a neutralization assay is used to diagnose SAV in some countries. RT-qPCR of heart is the most commonly used diagnostic method in Norway. Using whole virus particles disrupted with Triton-X in a bead-based immunoassay, antibodies against SAV were detected in both experimentally and naturally infected Atlantic salmon. When tested on plasma from PRV-infected Atlantic salmon, already known to produce high levels of non-specific antibodies, no background binding to the SAV-coated beads were detected. This indicates that the antibodies detected are specific. The levels of SAV-specific antibodies were strongly correlated with virus neutralization titers. This assay is time-saving compared to neutralization assays, can detect infected fish for a longer time period than RT-qPCR and is compatible with non-lethal sampling.

4 RESULTS

4.1 ANTIBODIES AGAINST PRV

In the first paper, we aimed at detecting antibodies against PRV-1 proteins µ1c, µNS, σ1 and σ3. In order to do so, recombinant proteins were made and coated on MagPlex beads to be run in the xMAP bead-based multiplex immunoassay. The outer core proteins µ1c, σ1 (Finstad et al., 2012) and σ3 (Markussen et al., 2013) were made in *E.coli*, and the non-structural µNS protein was made in the baculo/insect cell system. Supernatant from a centrifuged lysate of untransfected *E. coli* and uninfected sf9 insect cells were coated on beads and used as background controls. A PRV-1 challenge trial was performed on Atlantic salmon parr in freshwater at VESO Vikan aquatic research facility. Naïve fish were i.p. injected with 0,1ml of a PRV positive inoculum consisting of pelleted blood cells collected in a previous cohabitation trial. Antibodies against PRV-1 protein µ1c and µNS were detected in plasma from cohabitant fish using the xMAP method (figure 11). The corresponding background was then subtracted from the results. Very low background was detected except in one fish at 10 wpc where the MFI from the *E. coli* background bead exceeded the MFI from the µ1c beads.

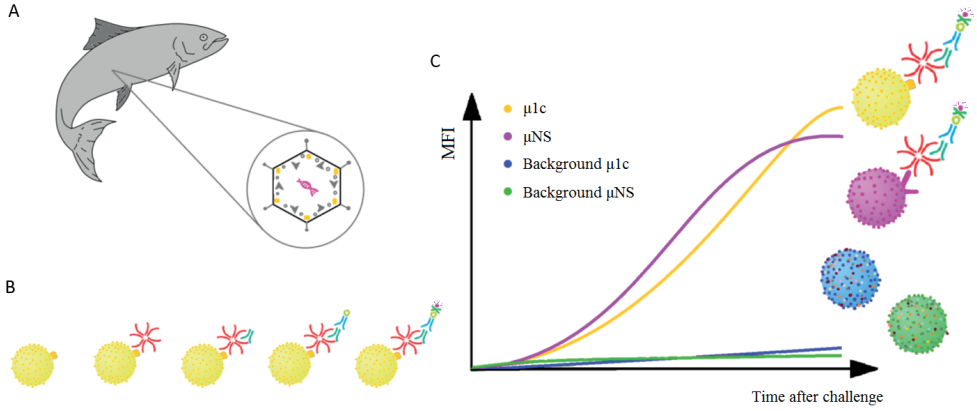

Figure 11. *Summary of results from paper I. Atlantic salmon infected with PRV (A), step-wise illustration of binding with IgM from salmon plasma, anti-salmonid antibody, secondary antibody and streptavidin-PE to bead coated with proteins (B) and antibody level development over time (C).*

Antibody levels increased in cohabitating fish from 6 weeks after the introduction of infected shedders (figure 12). For µ1c-targeted antibodies, the levels continued to increase through the duration of the challenge (10 weeks). Antibodies against µNS seemed to reach a peak level at 8 weeks. Infection was confirmed through PRV RT-qPCR and pathology consistent with HSMI was

found in hearts from 6 weeks. Histopathological changes in the heart and virus RNA levels decreased from around 8 weeks, when antibody levels were rising or plateauing. Western blotting confirmed antibodies binding to µ1c but failed at detecting antibodies against µNS.

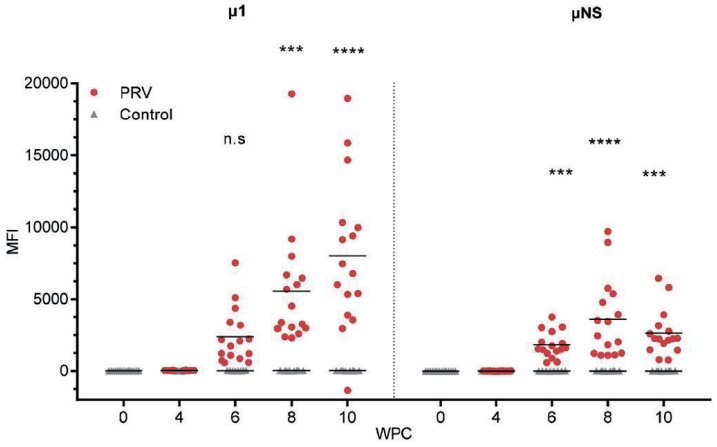

Figure 12. *Kinetics of PRV µ1c- and µNS-specific IgM production (from paper I).*

No antibodies against σ1 or σ3 were detected. σ1 and σ3 are both outer capsid proteins and σ1 is the spike protein in PRV. In MRV, the σ1 protein attaches to cell surface receptor JAM-A and is important for the cell and tissue tropism (Weiner *et al.*, 1977, Lee *et al.*, 1981, Barton *et al.*, 2001). We would therefore expect the infected fish to produce antibodies against these proteins. We concluded that the most likely reason we did not detect such antibodies was that the recombinant proteins made in *E. coli* were not recognized as the native protein by the antibodies, possibly through aberrant folding or less accessible epitopes when coated on the beads.

In paper II, a new method was therefore used when producing recombinant σ1 in *E. coli*. By modifying the N-terminal end of the recombinant PRV-1 σ1 with a lipid residue, antibodies towards σ1 became detectable in the xMAP assay. The anti-PRVσ1 antibodies were detected in plasma from fish in a PRV cohabitation challenge lasting 15 weeks. This challenge was performed on seawater-adapted Atlantic salmon at VESO Vikan aquatic research facility as described earlier (Lund *et al.*, 2017). Antibodies started to increase in some fish from 7 wpc, coinciding with peak level of virus RNA in heart and blood, reached a plateau at 10 wpc, and stayed at the same level or slightly decreased towards the end of the trial (figure 13). Highest antibody levels were found at 10, 12 and 15 weeks. In this trial,

heart pathology was most pronounced at week 10. There were here, as well as in paper I, large individual differences. In contrast to what we found in paper I, we now found background IgM binding from plasma from control fish. After heat treatment of plasma at 48°C for 20 minutes, the MFI in samples from control fish was decreased, whereas the MFI reflecting PRV-specific IgM from infected fish stayed mostly unaffected (figure 13).

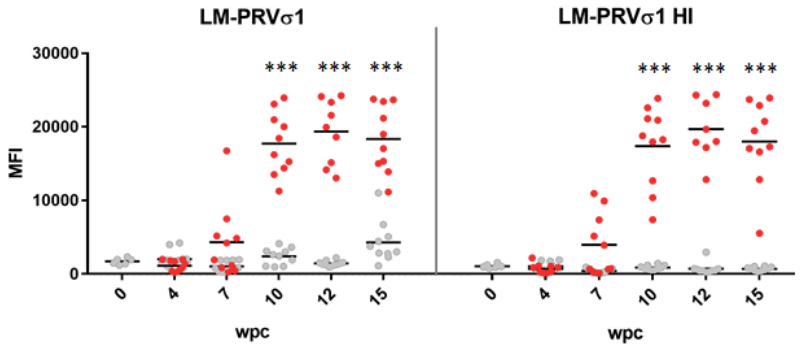

Figure 13. *Detection of antibodies against lipid modified σ1 in untreated and heat-treated (HI) plasma. Significant difference between controls (●) and cohabitants (●) indicated by asterisks (Mann-Whitney test) (from paper II).*

As controls we used other irrelevant proteins coated on beads; lipid modified ICP11 from the white spot syndrome virus (WSSV) (LM-ICP11), lipid modified F-protein from ISAV (LM-ISAV-FP) and recombinant F-protein from ISAV (ISAV-FP). We detected a high level of binding to all the control beads, mainly from PRV-infected fish (figure 14). This binding was reduced by heat treatment, but there was still a significant increase in MFI from cohabitants from 10 wpc (figure 14).

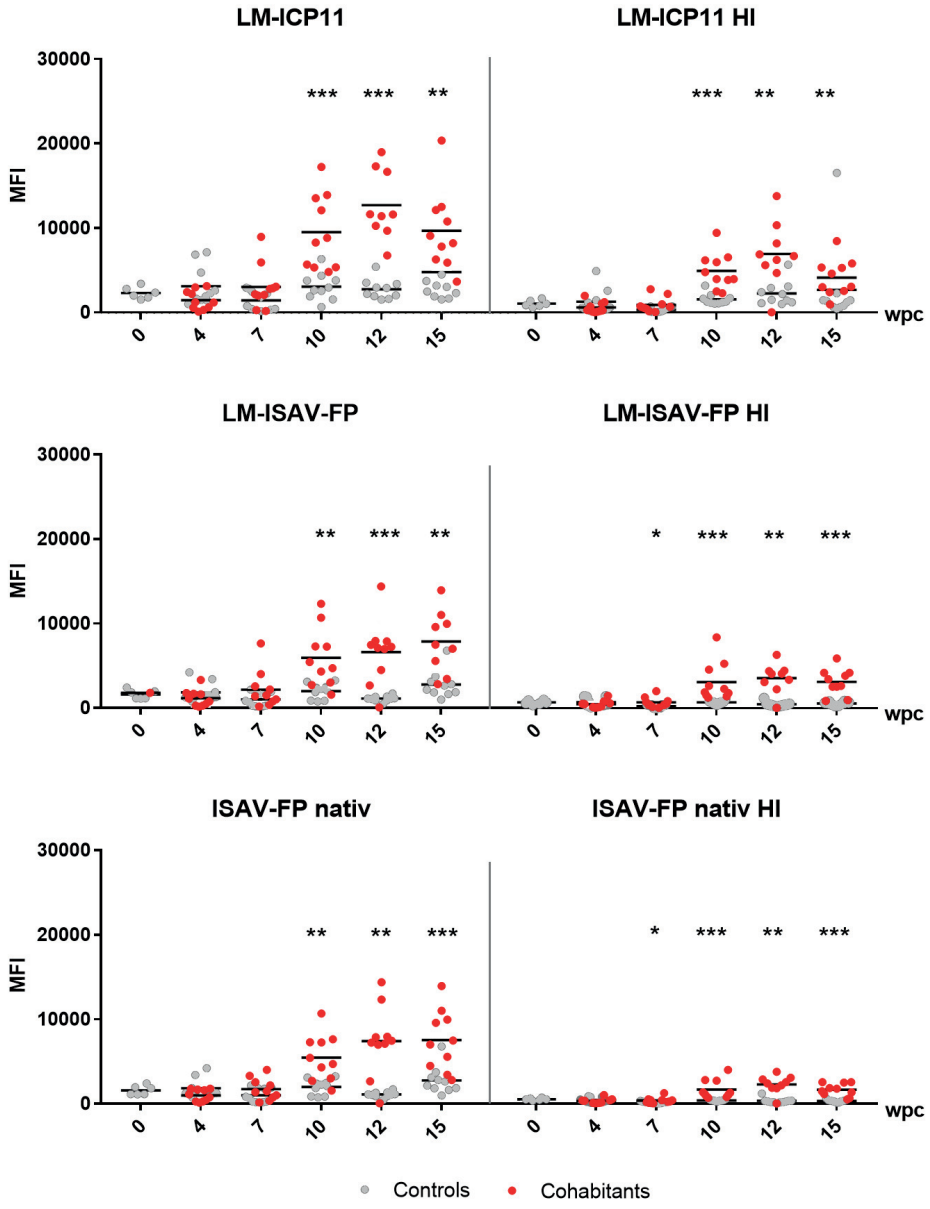

Figure 14. *Detection of antibodies against control proteins in untreated and heat-treated plasma. Significant difference between controls (●) and cohabitants (●) indicated by asterisks (Mann-Whitney test) (from paper II).*

Western blotting was used to confirm antibody specificity towards the lipid modified σ1 and the control proteins used, as well as the recombinant σ1 and µ1c used in paper I (figure 15). Only antibodies towards the two σ1 proteins and µ1c were detected when using plasma from PRV-infected fish as primary antibody on the blot. No antibody binding to any proteins were detected when plasma from control fish was used.

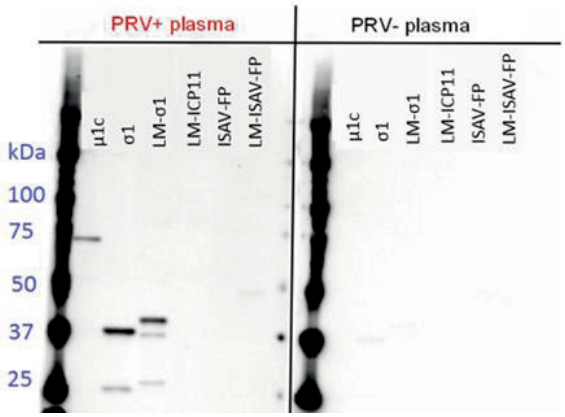

Figure 15. *Western blot showing IgM antibodies against PRV proteins used in the multiplex immunoassay. Left panel incubated with plasma from PRV-infected Atlantic salmon (pooled sample from 10 to 15 wpc) and right panel incubated with plasma from control fish (pooled samples from the same challenge trial) as primary antibody (from paper II).*

To further evaluate the specificity of both the lipid modified σ1 and the control proteins, adsorption against coated beads (40 times as many beads as is used in the assay) with the different antigens were performed, and then analyzed as normal in the xMAP assay (figure 16).

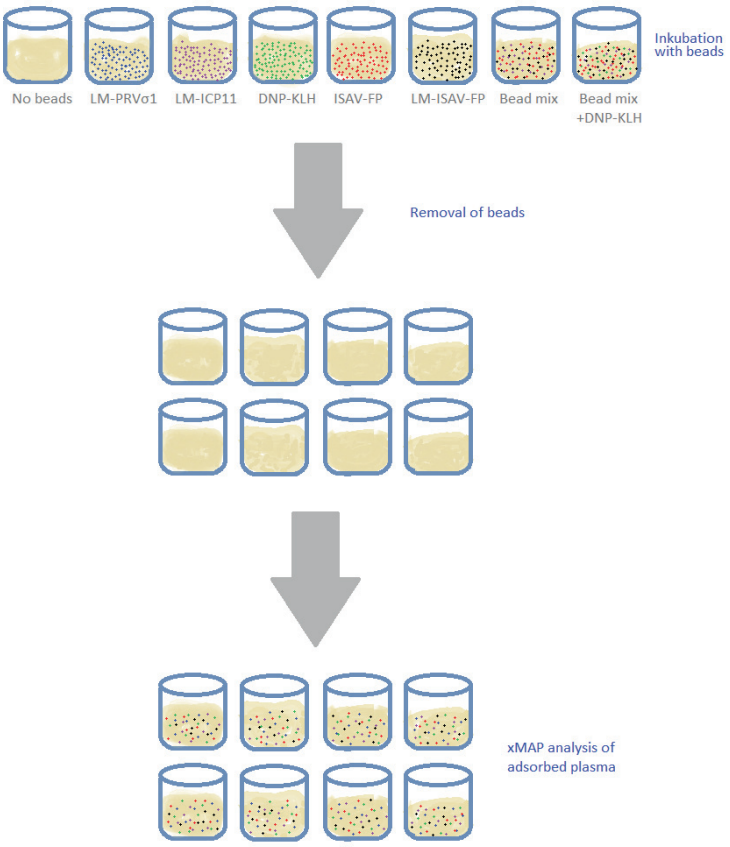

Figure 16. *Adsorption of plasma followed by removal of beads and xMAP analysis.*

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Results from the adsorption analysis indicate that the antibodies binding to σ1 beads were specific (figure 17). Binding to beads with LM-PRV-σ1 was not reduced after adsorption against any other antigens than itself. Binding to the beads with irrelevant antigens were not reduced by binding the other irrelevant antigens and only by around one third after pre-adsorption against themselves. They were however reduced to roughly half after pre-adsorption against a bead mix containing DNP-KLH, which has been used to measure polyreactive antibodies, indicating that these antibodies are polyreactive.

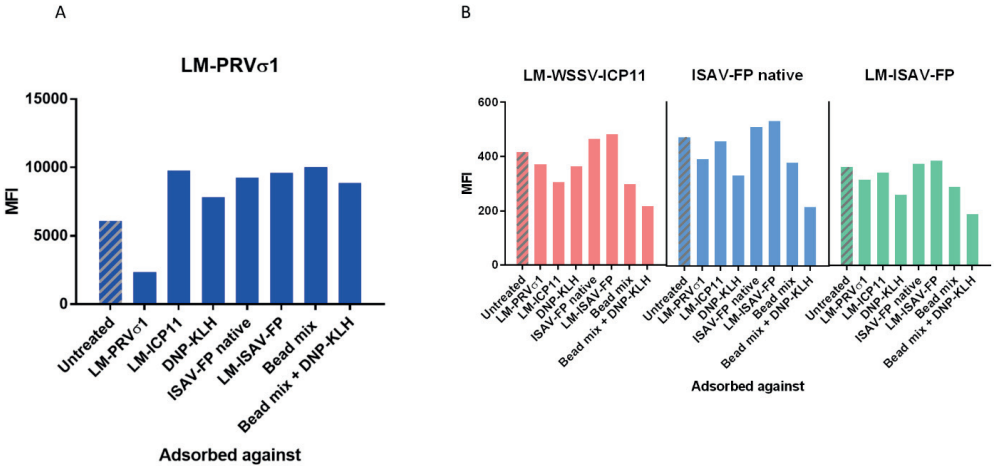

Figure 17. *Pre-adsorption of heat-treated pooled plasma from PRV-infected fish against antigens indicate specificity of anti-PRVσ1 antibodies (paper II). Measured on beads coupled with (A) LM-PRVσ1 and (B) non-PRV control proteins. (Bead mix: LM-WSSV-ICP11, LM-ISAV-FP and ISAV-FP)*

4.3 ANTIBODIES AGAINST SAV

In paper III, we wanted to use the xMAP technology to detect anti-SAV antibodies. This could be a valuable supplement in PD diagnostics and surveillance. Several recombinant SAV proteins, including a recombinant SAV3 membrane protein E1 made in *E. coli*, a lipid modified E1 also made in *E. coli* and purified SAV-protein E2E3 made in the baculo/insect cell system had been previously tested, but failed at detecting any antibodies when used as antigen on the beads (unpublished). We now tested a recombinant SAV3 E2 made in *E. coli* and whole SAV particles from SAV3-infected CHSE cells that were stably transfected with the structural SAV1 cassette.

Plasma from a SAV2 and SAV3 challenge trial was used to detect antibodies against SAV with the xMAP assay. The trial was performed on post-smolt Atlantic salmon at VESO Vikan aquatic research facility (Lund et al., 2016) This trial originally investigated SAV and PRV co-infections, but only SAV-positive control fish (not infected by PRV) were used here. RT-qPCR, histopathology and virus neutralization tests from this challenge trial had been published previously (Lund et al., 2016, Rosaeg et al., 2017). SAV RNA levels increased from week 2, and heart and pancreas pathology was most pronounced from week 3. Both virus RNA and heart lesions were decreased in week 6, whereas pancreas pathology remained extensive throughout the 6 weeks the trial lasted. Neutralization tests also showed neutralizing antibodies in all fish sampled at 6 weeks and in some fish from earlier time points.

Beads with whole SAV virus particles produced in CHSE cells were coated on beads (SAV 1:1) and used to analyze heat-treated plasma from the challenge trial. The MFI, reflecting IgM binding to the beads, was generally low, but with a clear discrimination between the early weeks and 6 wpc (figure 18). To improve the whole virus assay, the virus particles were disrupted by pre-treatment with Triton-X before coating on beads (SAV-TX). A SAV E2 protein made in *E. coli* was also coated on beads (SAV-E2) and both beads were tested in the assay. The SAV-E2 beads showed increased IgM binding from week 6 after challenge (figure 18). The Triton-X treatment resulted in improved antigen recognition when using the same number of virus particles as the untreated whole virus beads. When the SAV-TX and SAV-E2 beads were tested on SAV-negative, PRV-infected fish from a challenge trial, we found high levels of binding to the SAV-E2 beads. However, no background binding to SAV-TX was observed.

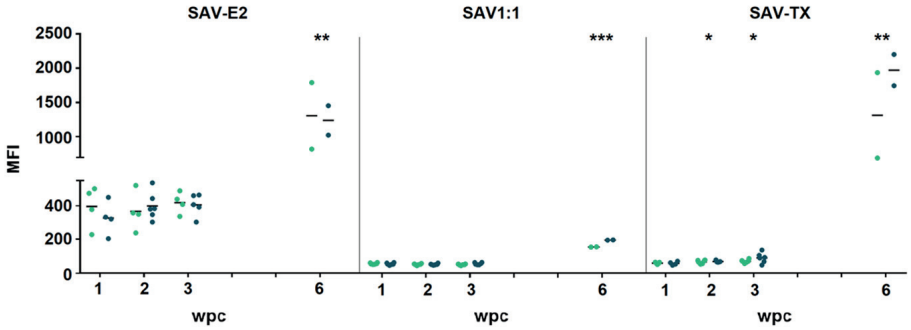

Figure 18. *Detection of antibodies against SAV2 (•) and SAV3 (•) using E2 and disrupted virus particles as antigen (paper III). MFI with mean values indicated by lines. Significant differences compared to 1 wpc are indicated by asterisks (Mann-Whitney test).*

Virus neutralization assays are sometimes used to assess antibody formation against SAV and can be used to detect a SAV infection. These assays are time-consuming to perform, and an xMAP assay could be a good alternative. We therefore tested the xMAP assay on field samples from a PD outbreak (pen number 10) caused by SAV2 (Rosaeg *et al.*, 2019).

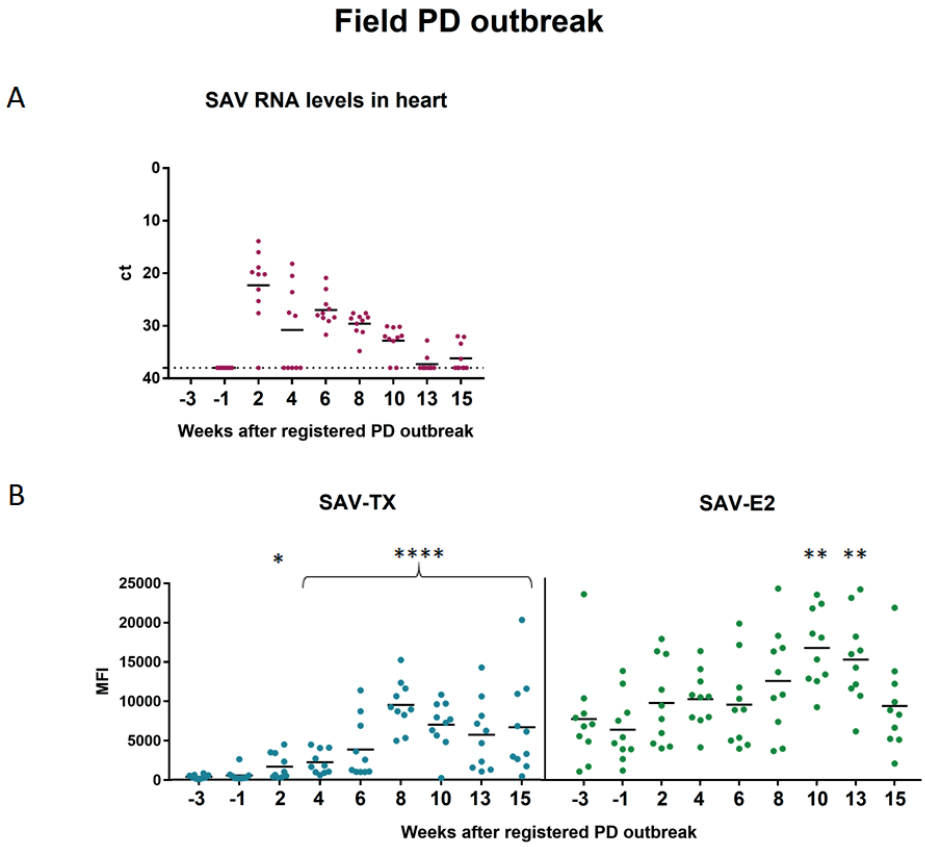

Figure 19. *A) SAV RNA levels in hearts from fish in a field outbreak of pancreas disease caused by SAV2. Mean values indicated with lines. B) Levels of antibody binding to SAV-TX and SAV-E2 coated beads from plasma from the same outbreak. Mean values indicated with lines. Significant differences compared to three weeks before the outbreak are indicated by asterisks (Mann-Whitney test).*

Viral RNA was first found using RT-qPCR the second week after the PD outbreak (figure 19a). The results from the xMAP showed increasing antibody levels binding to SAV-TX from around two weeks after the outbreak which stayed elevated until the last sampling point at 15 weeks (figure 19b). Binding to SAV-E2 showed a similar pattern, but with high values already prior to infection, and higher variation between individuals (figure 19b)

The SAV ct values obtained by RT-qPCR were mainly undetected from week 13, in contrast to the SAV-TX binding antibodies, (figure 19). This shows that serology could be used to detect previously SAV-infected fish or fish populations for a longer time period than RT-qPCR.

The results from the xMAP analysis was compared with results from virus neutralization (figure 20). A strong correlation between neutralization titers and MFI from SAV-TX beads was found (Spearman's ρ = 0,73, p<0,0001).

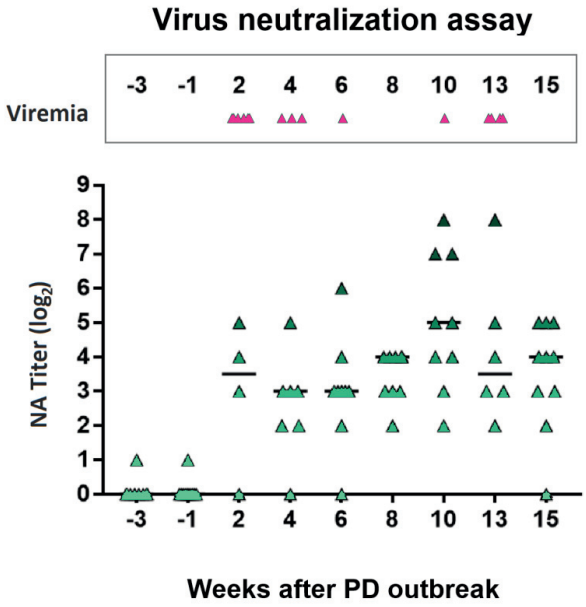

Figure 20. *Neutralization titres, with median values indicated by lines, from PD field outbreak. Viremic samples in pink.*

4.5 UNPUBLISHED WORK – RESULTS AND DISCUSSION

As is often the case, this PhD work has deviated and changed from the original plan. In the following chapter, I present results from experiments carried out during this project that did not reach conclusive results or that could not be pursued for different reasons. I believe they nevertheless may provide valuable information for future efforts.

4.5.1 Ambient analyte theory

Results from the bead-based immunoassay we have used should, according to the ambient analyte theory, be independent of the sample volume, and the concentration of analyte in the sample should not be affected by the analysis. Two tests were conducted to verify this. First, one, two, three or four consecutive incubations with beads and the same plasma were performed, identical to the first step in the xMAP protocol. Samples from the same fish were incubated one, two or three times. After a 30-min incubation period, beads were removed from the sample, and new beads were added. This procedure was repeated three times. Finally, all three plasma samples as well as an untreated sample from the same fish were analyzed with the xMAP protocol described in the papers and read with the Bio-Plex 200 array reader. The plasma was from the same challenge study as in paper II, and two fish from 12 wpc and two control fish were used. The average reduction in MFI from one incubation to the next was around 5 % (figure 21A). This was in line with what Poetz et al found when using xMAP to test sequential multiplex analyte capturing in a sandwich immunoassay (Poetz et al., 2010). Secondly, different volumes of the same sample were used: 100 µl, 75 µl, 50 µl and 25 µl. The samples were from one fish at 15 wpc from the same challenge, and the sample dilutions were run in duplicates. Only minor changes were observed between 50-100 µl, but 25 µl gave slightly lower MFI values (figure 21B). When washing the beads before adding the sample there will always be some fluid left from the washing buffer. This amount of a few microliters will obviously affect the concentration of the smaller samples more than the larger samples, and this is likely what caused the reduction of MFI in the 25 µl samples.

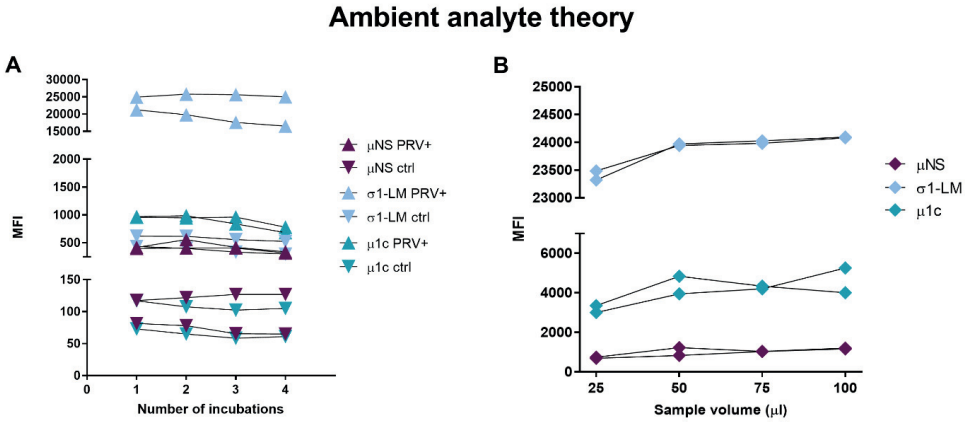

Figure 21. *Demonstrating the ambient analyte theory, showing that the results are not significantly affected by changes in number of incubations (A) or sample volume (B).*

4.5.2 PRV-neutralizing antibodies

Measuring antibody binding in PRV-infected fish, as we have done in our papers, provide valuable information, but virus neutralization assays reveal more about the antibodies' actual effect on the virus. Antibodies against σ1 in MRV are shown to be neutralizing (Tyler *et al.*, 1993). If this is also the case with PRV has not previously been investigated. There is no available cell line suitable to propagate PRV, but the virus can replicate in erythrocytes *ex vivo* and are detectable by flow cytometry and RT-qPCR (Wessel et al., 2015b). We therefore set up an assay to assess the ability of defined serum samples to prevent PRV infection of erythrocytes *ex vivo*.

Erythrocytes from healthy Atlantic salmon were isolated with a percoll density gradient and kept in Leibovitz's L-15 Medium (L-15) (Thermo Fisher, Waltham, MA, USA) containing 50 µg/ml Gentamicin (Thermo Fisher) and 2% Fetal calf serum (FCS) in 24 well plates overnight with rotation. 3 x 10^8 particles of purified PRV (Wessel et al., 2017) in dPBS were incubated with a 1:20 or a 1:40 dilution of plasma from either uninfected or PRV-infected (week 12 post PRV challenge) fish for two hours before 10 µl (3 x 10^7 PRV particles) were added to each erythrocyte well. Negative control wells were added only dPBS, and positive control wells were added 3 x 10^7 PRV particles in dPBS. After 24h at 15°C and 120 rpm, all erythrocytes were washed twice with L-15, followed by the first sampling (1 dpi). Parallel wells were added fresh medium and incubated further (15°C with 120 rpm) until 7 and 13 dpi. Upon each sampling, erythrocytes from each well were transferred to Eppendorf tubes,

washed in 1 ml PBS, lysed in 500 µl QIAzol Lysis Reagent (Qiagen), and stored at -20°C for further RNA isolation. At 13 dpi parallel samples were also harvested for flow cytometry.

No erythrocytes in any samples were shown to be PRV positive using flow cytometry with polyclonal rabbit anti-PRV as primary antibody (not shown). RNA was isolated from 13 dpi samples, cDNA synthetized and then analyzed by RT-qPCR. cDNA corresponding to 10 ng RNA was analyzed in triplets for 40 cycles of 94 °C/15 sec, 60 °C/30 sec. Levels of the reference gene elongation factor 1 α (Ef1) and the antiviral response gene viperin-1 mRNA were assessed using 500 nM primers and the Maxima SYBR Green/ROX qPCR Master Mix (Fisher scientific). Primers used are previously published (Dahle et al., 2015). The specificity of the SYBR Green assay was confirmed by melting point analysis. Levels of Ef1 mRNA were used for normalization (Lovoll *et al.*, 2011).

Negative control erythrocyte samples were added dPBS only, positive control samples were added virus that had not been incubated with plasma. Samples called “Neg. plasma” were added virus incubated with plasma from virus negative fish, and samples called “Pos. plasma” were added virus incubated with plasma containing anti-PRV-IgM. The presence of neutralizing antibodies in “Pos. plasma” should then result in reduced infection and less up-regulation of viperin genes compared to the positive control and “Neg. plasma”.

Results using normalized ΔΔct values were not significantly altered (figure 22A) but could indicate a protective effect of plasma from both control fish and infected fish. When looking at un-normalized ct values, there were slightly higher viperin levels in the positive control than in the other groups (figure 22B). Samples from positive plasma had a slightly lower level, especially one sample. However, no significant differences were found between the groups.

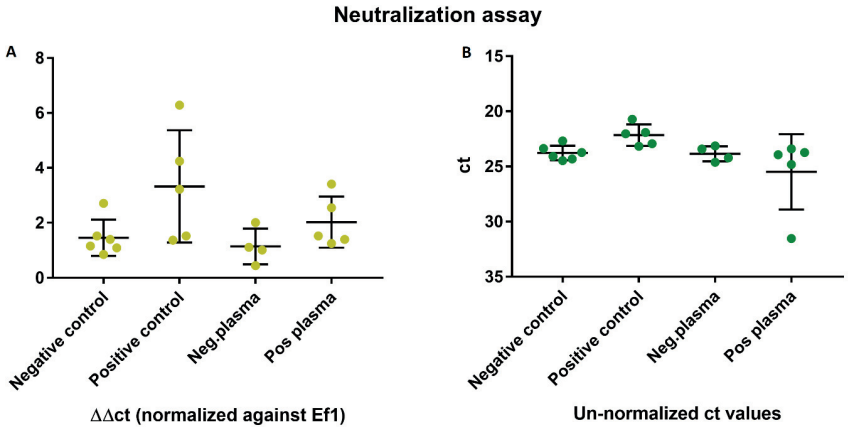

Figure 22. *Showing values of viperin-1 in a neutralization assay. A: Normalized (against elongation factor 1α) Data show relative expression (fold to control). B: Un-normalized ct values.*

A likely reason for the inconclusive results is a lack of efficient PRV infection, since erythrocyte infection ex vivo is not a reliable system. If found, a cell line suitable for PRV propagation could replace erythrocytes to investigate if antibodies towards PRV are neutralizing.

4.5.3 IgM/IgD-positive B cells

To describe the B cell response during an infection, one should first know the distribution and features of B cell populations in healthy individuals. Because B cell populations in Atlantic salmon had not been described prior to this project, we aimed to characterize different B cell populations in salmon based on IgM/IgD expression. An anti-rainbow trout IgD antibody (Ramirez-Gomez et al., 2012) was available to us, and we wanted to see if it cross-reacted with Atlantic salmon. Spleens and head kidney from healthy juvenile Atlantic salmon (sea water phase, from the Institute of Marine Research's research facilities in Bergen) were mechanically homogenized and lymphocytes were isolated from these organs and from blood using a percoll density gradient. Cells were stained with live/dead staining, anti-rainbow trout IgD and the same anti-salmonid IgM antibody used in the immunoassays. Goat anti-mouse IgG1 Alexa Fluor 647 and goat anti-mouse IgG2a Alexa Fluor 488 were used as secondary antibodies. Cells were read on a BD FACSCanto II flow cytometer. Data was analyzed using the Kaluza software and gating was done on live cells and on a lymphocyte gate in the forward scatter/side scatter dot plot. Results show a population of IgD+IgM+ cells, a smaller population of IgD-IgM+ (J+-) cells and small numbers of IgD+IgM- (J-+) cells (figure 23).

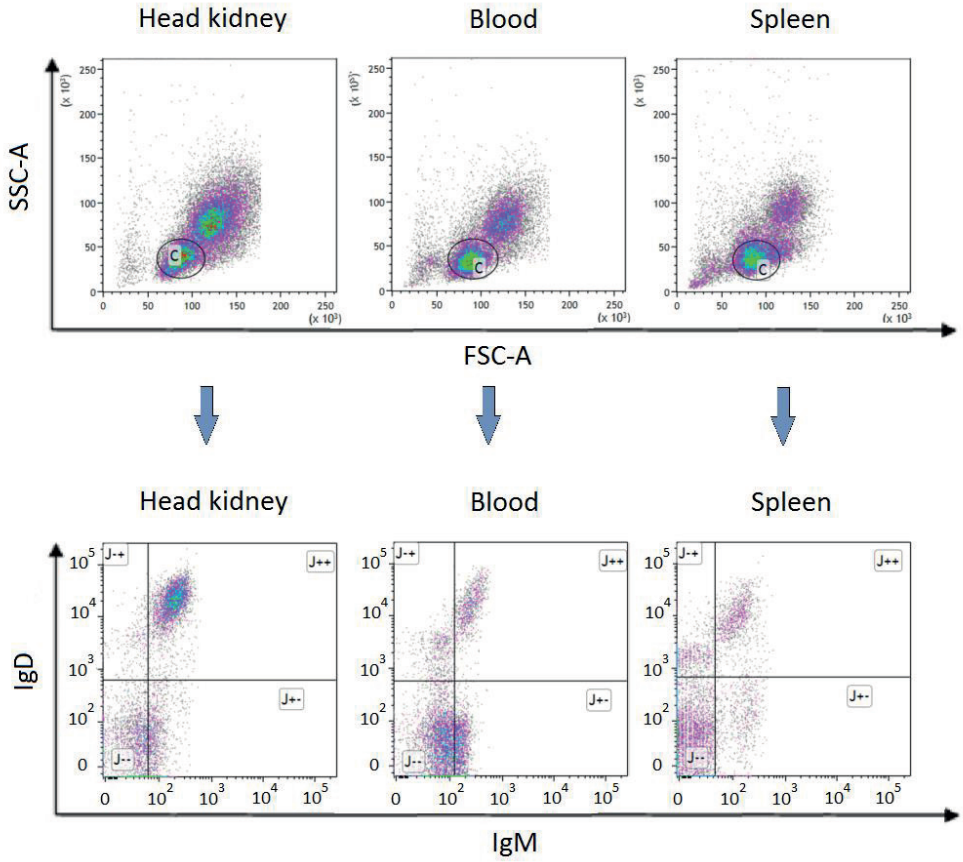

Figure 23. *IgM and IgD positive cells in head kidney, blood and spleen from healthy Atlantic salmon. Gating done on live cells and on a lymphocyte gate (C) in the forward scatter/side scatter dot plot. Cells read using BD FACSCanto II flow cytometer.*

Cells from blood from different fish (also from the sea water phase, from the National Institute of Water research's facilities at Solbergstrand) were later isolated, stained using the same protocol and analyzed with a different flow cytometer: the Gallios Flow Cytometer from Beckman Coulter. The same populations of cells are seen, but with a better separation of IgM negative and positive cells (figure 24).

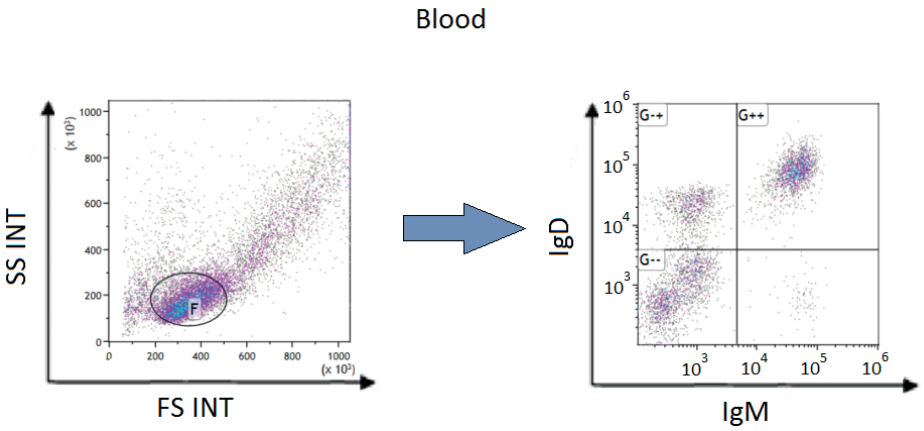

Figure 24. *IgM and IgD positive cells in blood from healthy Atlantic salmon using Gallios Flow cytometer.*

These findings of lymphocytes marking as IgD+, IgD+IgM+, as well as double negative, indicates a specific cross-reactivity of this anti-trout IgD antibody in the Atlantic salmon. The largest population of IgD+IgM+ cells were found in head kidney. This is in contrast to findings in rainbow trout where the largest population was found in blood, followed by spleen and then head kidney (Ramirez-Gomez et al., 2012). The head kidney is the place where naïve B cells are found (Bromage et al., 2004). These cells are double positive, coinciding with our results. The same population was also present in blood and spleen. This is expected, since mature naïve B cells migrate via blood to spleen and posterior kidney (Bromage et al., 2004). There seems to be IgD-IgM+ cells present mostly in spleen. These cells could be activated B cells (Parham & Janeway, 2015). They were also larger in size than the double-positive cells, supporting this. A population of IgD+IgM- cells is present in spleen and blood, but not in head kidney, and with less IgD than the double positive populations seen. IgD+IgM- cells have been described in kidney from rainbow trout with proliferative kidney disease (Abos et al., 2018b) and in gills and head kidney from both healthy and VHSV-infected rainbow trout (Castro et al., 2014a). Subsets of B-cells bearing these B-cell receptor variants should be more thoroughly studied in Atlantic salmon.

4.5.4 IgD in plasma

Small amounts of secreted IgD is found in rainbow trout plasma. The anti-rainbow trout IgD antibody used in flow cytometry was therefore used to look for PRV-specific IgD in plasma from the same

challenge trials as in paper I. No anti-PRV IgD antibodies above background were detected in plasma from fish (infected or controls) in the xMAP assay. When tested with western blotting, very small amounts (when compared with IgM) of IgD was detected in infected fish, but not in controls. The antibody could be suboptimal for xMAP, there could be too small amounts of IgD in plasma or it is possible that the amount of IgD increases in infected fish but do not bind to PRV μ1c. A study of rainbow trout detected very little IgD response and diversification following infection (Castro et al., 2013). Based on this and our results, we did not attempt to use the anti-IgD antibody further in xMAP analyses.

4.5.5 Cytotoxicity during PRV infection

Cytotoxic responses are considered important in the HSMI pathogenesis, since the myocarditis typical for HSMI is reported to be mediated by CD8 + cells (Mikalsen et al., 2012) and an upregulation of CD8 and granzyme genes have been observed in infected fish (Johansen et al., 2016). This up-regulation indicates that cytotoxic T cells are attacking infected cells. A PRV challenge experiment was set up to investigate cytotoxic responses with both gene expression, flow cytometry, antibody detection using xMAP and a functional assay to detect killing of infected erythrocytes by cytotoxic lymphocytes. Pre-smolt Atlantic salmon from a fresh water PRV-1 i.p. injection challenge trial at the Norwegian University of Life Sciences' aquarium facilities were used in this experiment. Infected fish were distributed between two tanks, tank A and tank B. Control fish were held in a separate tank. Heart, spleen, head kidney, blood pellet and plasma were sampled, and white blood cells were isolated from spleen using a density gradient.

The QuantiGene Plex assay (Thermo Fisher scientific) is a bead-based gene expression assay using techniques similar to the xMAP immunoassay. This assay was chosen as it is time saving compared to RNA isolation, cDNA synthesis and analysis with RT-qPCR. The potential for labor and time saving is better the more genes are analyzed, as one can multiplex up to 80 genes simultaneously from small amounts of sample material. In contrast to qPCR, no amplification of the nucleotide sequence is needed. Instead, the QuantiGene Plex assay uses branched DNA techniques with signal amplification to produce a fluorescent signal, as shown and described in figure 25. Around 30 000 cells, 50—500 ng cDNA or approximately 0,5 mg tissue is required in each sample.

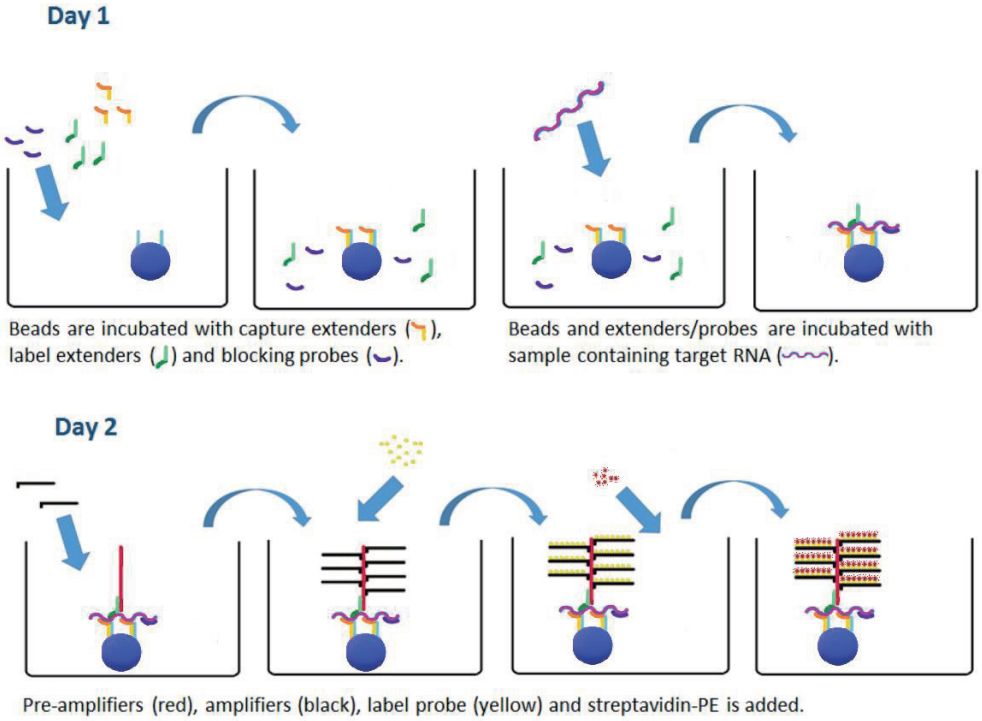

Figure 25 *Workflow and concept of QuantiGene Plex. Beads are coated with individual sequences. Custom-made capture extenders and label extenders are incubated with the beads, and one end of the capture extenders hybridize with the sequences on the beads. Then the sample is added, and the other end of the capture extenders binds to the target RNA sequence. One end of the label extenders hybridizes with a different sequence on the target RNA. A pre-amplifier sequence is added, and the other end of the label extenders binds to this. Amplifier sequences, biotinylated label probes and streptavidin-PE are added subsequently, and the beads are then analyzed in the same manner as in the immunoassay, with the Bio-Plex 200 and Bio-Plex manager.*

The three reference genes and 11 immune genes shown in table 2 were chosen for use in the QuantiGene assay, some because they were already offered by the producer, and some based on previous findings and our wish to look at cytotoxic activity.

Table 2. *Genes used in the QuantiGene Plex assay with accession numbers. Reference genes are marked in grey.*

Gene	Accession number
CD8α	NM_001123583
CD8β	NM_001123584
Mx1	NM_001139918
CD4	NM_001146408
IgM (membrane-bound)	XM_014203125
Interferon γ (IFNγ)	NM_001171804
Antimicrobial peptide NK-lysin precursor (Nkl)	NM_001141110
Perforin1.1	XM_014203336
Perforin1.2	XM_014156086
Granzyme A	NM_001141037
Granzyme K	XM_014133293
Elongation factor 1α (Ef1)	NM_001123629
Ribosomal protein S20 (Rps20)	NM_001140843
Eukaryotic translation initiation factor 3 subunit E-A (Eif3ea)	NM_001141695

White blood cells from spleen were isolated using a percoll density gradient. Tissue samples and cells were pre-treated according to the manufacturer's instructions. Briefly, heart and spleen samples were treated with homogenizing solution containing proteinase-K, shaken with a steel bead in a tissue lyser and incubated at 65 °C. Cells were incubated with lysing solutions containing proteinase-K at 50-55 °C. First samples from two PRV-injected and two control fish from week 6 in a PRV challenge trial were used to test the method. For comparison, RNA was isolated from the same samples. Different sample concentrations from both tissues, cells and isolated RNA were analyzed to test and optimize the method.

This initial test was successful for both cells, tissue and RNA. Linear titration curves were found for all genes. IgM and the reference genes Ef1 and Rps20 reached the maximum limit of detection in many samples, especially from spleen tissue. For heart tissue, the values were generally much lower. Detection levels were also higher when using tissue lysates compared to using the corresponding isolated RNA. There were some differences in gene expression between infected and control fish, but also between the two infected fish tested. For the main analysis of the rest of the samples, it was decided to not isolate RNA, but only use tissue or cells lysates. A decision to use Eif3ea as the normalization gene was made based on the finding that rsp20 was around the maximum limit of detection in most samples and that Ef1 was up-regulated in blood in PRV infected fish. Up-regulation of Ef1 in infected erythrocytes has also been observed by others (Rosaeg et al., 2017). It was also

decided to use less tissue than in the initial test when analyzing spleen samples and more cells when analyzing blood and gradient isolated cells.

Then heart and spleen from all fish at four selected time points were tested in the assay, but this time we not detect any values above background levels in any of the samples. The explanation for this was not found. Blood pellets and isolated white blood cells were tested next. This time, we did not manage to fully lysate the cells from the blood pellet, most likely because we had increased the cell concentration too much. The solution ended up too viscous to analyze. The analysis of isolated white blood cells from spleen did give results, as shown in figures 26 and 27.

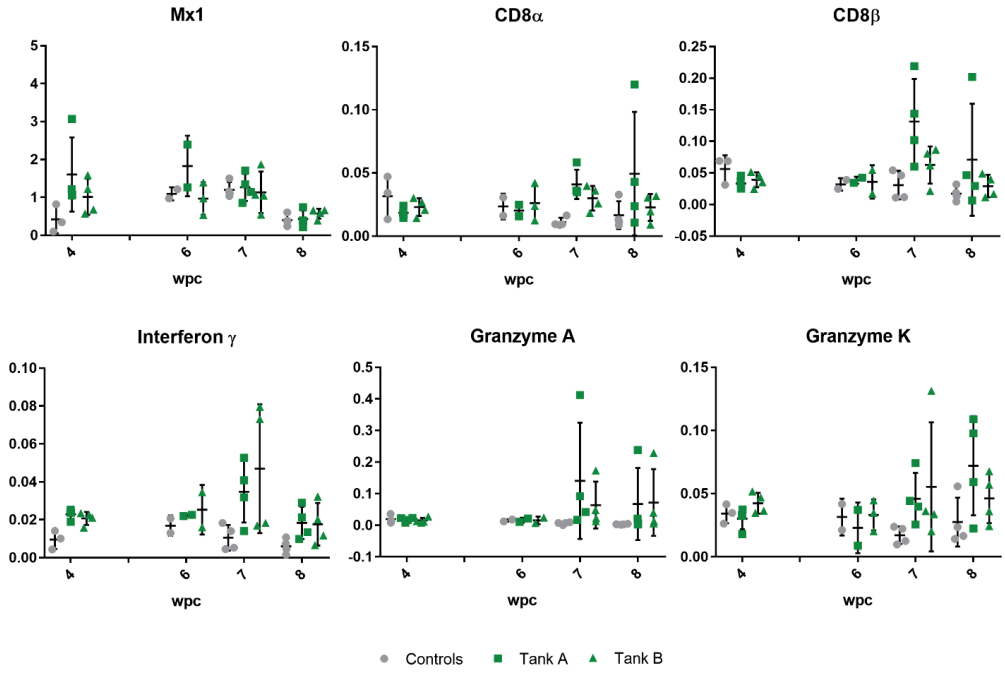

Figure 26. *Expression of immune genes normalized against Eif3ea. Mean with SD. Tank A and B are parallel tanks with PRV infected fish. wpc = weeks post challenge.*

Granzyme A showed the most consistent values in all control fish (figure 26 and 27). For the other genes, there were larger individual variations. When looking at relative expression (figure 27), Mx was up-regulated between 2-7-fold at 4 wpc but not at later time points. Mx is a GTPase with antiviral activity, and is induced by interferon in salmon, more strongly by interferon type I than by interferon γ (Sun *et al.*, 2011). Interferon γ was up-regulated primarily at 7 and 8 wpc, but also slightly before

this. IFN-γ is reported to have antiviral activity against IPNV in Atlantic salmon cells, and, like PRV, IPNV is a naked double-stranded RNA virus (Sun et al., 2011).

The cytotox-related genes CD8α, CD8β and granzyme K were up-regulated two to 10-fold at 7-8 wpc (figure 27). Granzyme A was up-regulated up to more than 60-fold in a few fish at 7-8 wpc and interferon γ was also up-regulated at 7 and 8 wpc (figure 27). NKL, IgM, perforin 1-1 and CD4 also tended to be up-regulated at 7 and 8 wpc, but only up to three-fold and with large individual differences. No up-regulation of perforin 1-2 was observed (data not shown).

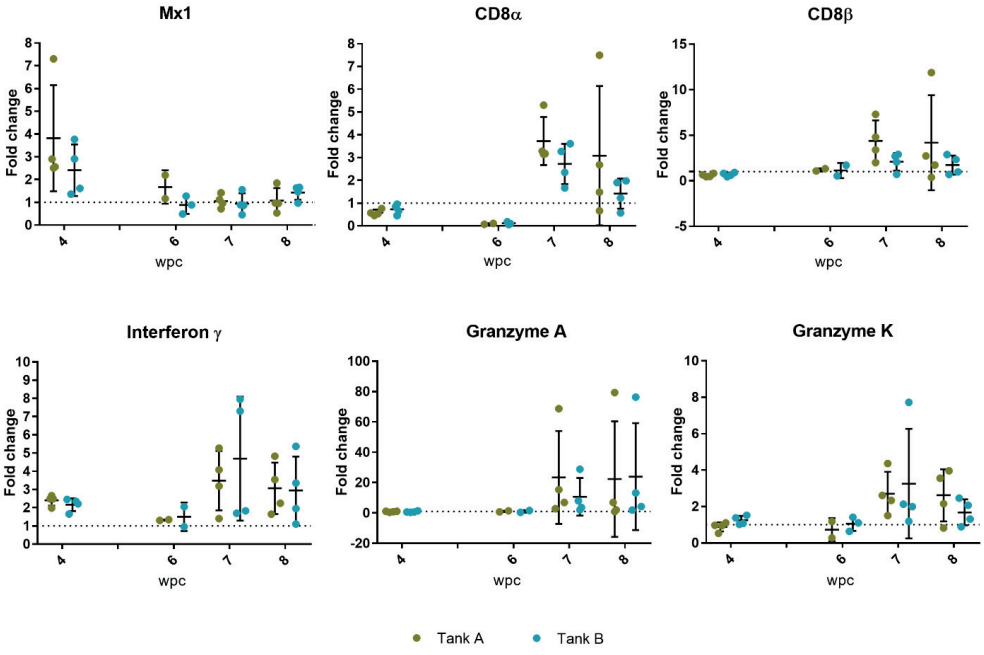

Figure 27. *Fold change of immune genes compared to mean of the control group at each time point. Mean with SD. Tank A and B are parallel tanks with PRV infected fish. wpc = weeks post challenge.*

Virus level was determined by RT-qPCR, using PRV-specific primers, from blood pellet (figure 28). A high and statistically significant correlation between low virus level and high gene expression was found with CD8α ($r^2 = 0,73$, $p = 0,0067$), CD8β ($r^2 = 0,73$, $p = 0,0068$) and CD4 ($r^2 = 0,85$, $p = 0,001$) at 8 wpc. A lower correlation was also found between high virus levels and high gene expression of Mx ($r^2 = 0,584$, $p = 0,036$) and interferon γ ($r^2 = 0,58$, $p = 0,028$) at 7 wpc. The fish with the lowest virus level in tank A from 8 wpc (figure 28) is the same fish that had the highest expression of CD8α,

granzyme A, CD8β (figure 27) and of CD4 (data not shown), and also the fish with the highest number of CD8 positive cells detected by flow cytometry (figure 29).

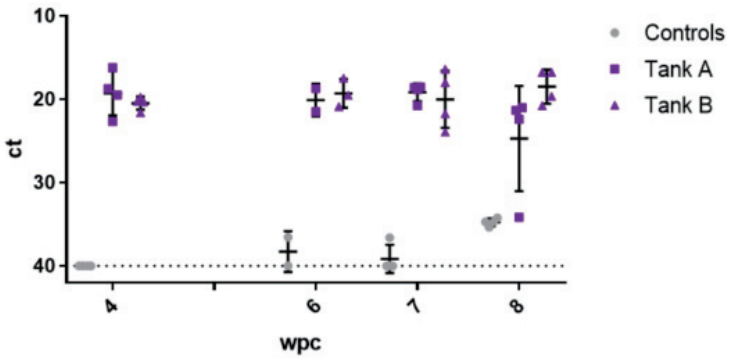

Figure 28. *PRV RNA levels in blood pellet from control fish (grey) and infected fish from two tanks (purple). Mean with SD.*

Had the method been successful also for heart and spleen samples, the results would have been compared to RT-qPCR data of gene expression from the same samples. Unfortunately, no sample material was left to reanalyze, and repeated studies could not be done due to financial restrictions. Nevertheless, our results indicate that this method has a potential for use in gene expression analysis also in Atlantic salmon.

In addition to the QuantiGene plex assay, flow cytometry was used to analyze the density gradient isolated cells from spleen. Antibodies against the markers salmonid IgM, rainbow trout CD8α (Takizawa *et al.*, 2011) and rainbow trout CD3ε (Boardman *et al.*, 2012) were used. Anti-mouse IgG2a APC (Allophycocyanin), anti-rat Ig PE and anti-mouse IgG1 Alexa Fluor 488 were used as secondary antibodies. Analysis were performed using the Gallios Flow Cytometer. For the first time points, we found very low to non-existing levels of CD8 positive cells. At 7 wpc and 8 wpc, the numbers increased substantially. However, we had to conclude that this increase was due to an unfortunate change to a new batch of the secondary antibody anti-rat PE in the middle of the trial. It is very likely that the staining properties differed between these batches. Since repetition of the in vivo trial was not feasible, we chose to dismiss data from the outdated batch used for the early time points and only consider week 7 and 8. In addition to increased binding, we found much unspecific binding to CD3

negative cells from this new batch of secondary antibody. These other cells turned out to be IgM positive (dark blue in figure 30) when stained with the anti-salmonid IgM antibody described earlier.

The CD3ε marker was expressed on between 1,4 - 4,7 % of lymphocyte gated cells in control fish and 3,1 – 10,9 % in infected fish from 7 wpc and 8 wpc. There was a small population of CD3+CD8+ cells, which represented less than 0,25 % of lymphocytes, in all fish. An increase was seen in infected fish compared to controls, but only statistically significant in tank A at 8 wpc (figure 29). Representative plots of analyzed cells from control fish (A) and PRV-infected fish (B) are shown in figure 30. The portion of lymphocytes that was stained with this anti-CD8 antibody is lower than in rainbow trout, where around 2 % of splenocytes were positive (Takizawa et al., 2011). It should be stressed that this antibody was raised against rainbow trout CD8. Our results may indicate a low affinity of this antibody to the Atlantic salmon CD8, emphasizing the need to fully verify it for this species.

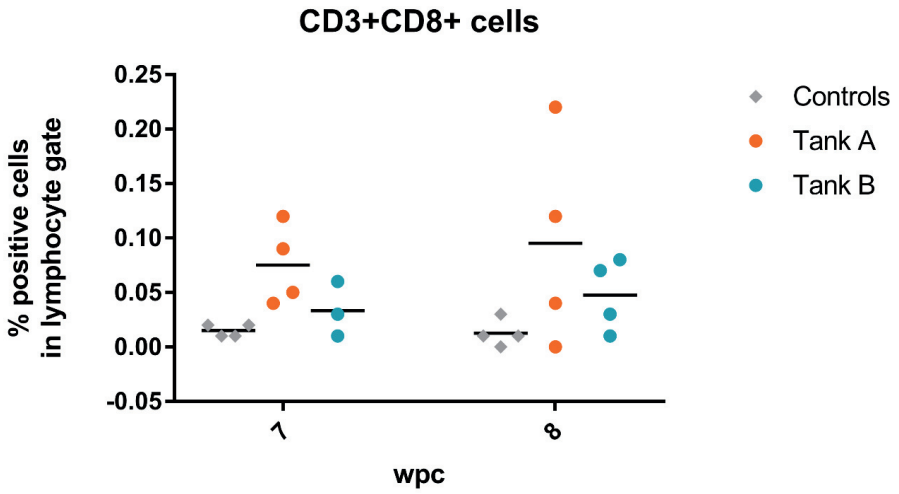

Figure 29. *Fraction of CD3+CD8+ lymphocytes from spleen of control and infected fish at 7 and 8 wpc in a PRV challenge trial. Mean values indicated with lines.*

Nevertheless, the number of CD3+CD8+ cells measured using flow cytometry had a high and statistically significant correlation with CD8α expression measured in the QuantiGene plex assay ($r^2 = 0,73$, $p < 0,0001$). The correlation with CD8β expression was lower ($r^2 = 0,57$, $p < 0,0001$).

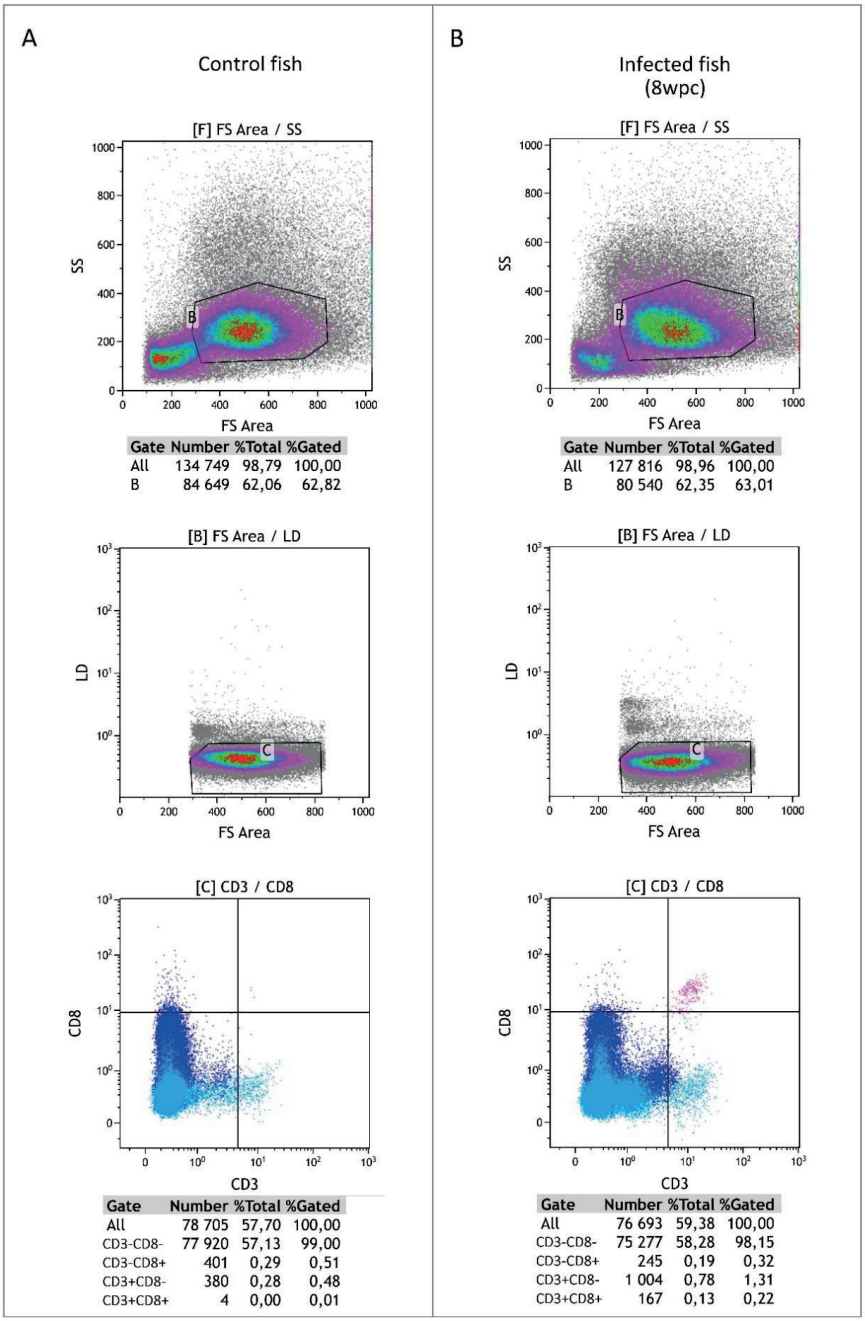

***Figure 30**. Representative plots of density gradient isolated cells from spleen of control fish (A) and PRV infected fish (B) from 8 wpc analyzed with flow cytometry. Gating was done on lymphocyte-like cells in a FS/SS plot and on live cells in a FS/LD (Live/Dead staining) plot. A population of CD3+CD8+ cells are present in PRV infected fish at 8 wpc. Dark blue cells in the CD3/CD8 plot are IgM positive cells.*

The IgM+ cells with apparently increased CD8 detected in this experiment were likely just unspecific binding to the IgM+ cells by the secondary antibody against the CD8+ cells. There seemed to be a population of CD3+IgM+ cells in some of the fish, especially in infected individuals. This could be a flow cytometry artefact caused by doublets (Henry *et al.*, 2010), more un-specific binding by secondary antibodies or perhaps a transfer of markers between cells before analysis (Nagel *et al.*, 2014).

The xMAP method was used on plasma from all fish and showed increased levels of antibodies against LM-PRVσ1 beads at week 6, 7 and 8 in infected fish (figure 31).

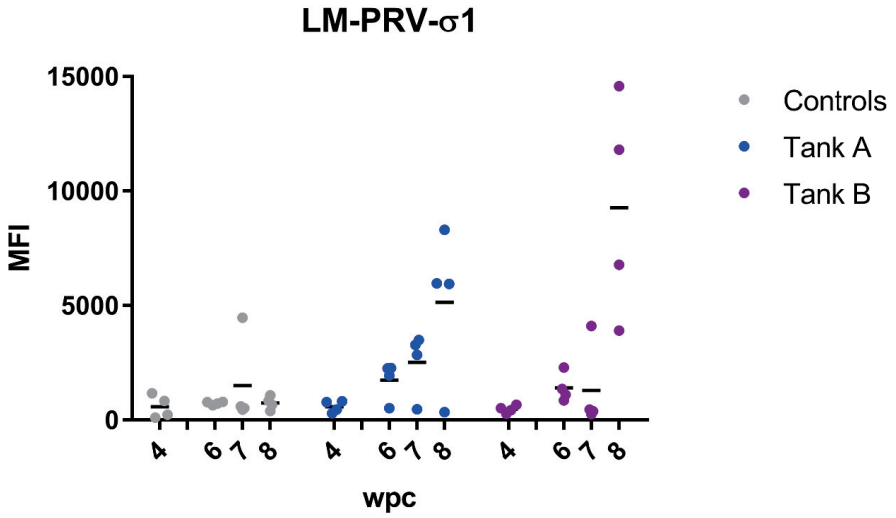

Figure 31. *xMAP analysis of plasma showing increasing antibody levels from 6 wpc. Mean values indicated with lines.*

Two cytotoxicity assays were also tested to quantify cell death. Isolated erythrocytes were incubated with isolated blood leucocytes from the same fish. The goal was to find differences in cell-mediated killing in infected and control fish. First the vybrant cytotoxicity assay (Molecular probes) measuring the amount of glucose-6-phosphate dehydrogenase in the supernatant was tested. This is an enzyme present in all cells, but with the highest concentration in more metabolically active cells. Since the target cells were erythrocytes with low metabolic activity, the vybrant assay was not sensitive enough. Lots of hemoglobin was released to the supernatant when the erythrocytes were lysed, and this may have also disturbed the reading of the vybrant assay, perhaps along with the pink color of the cell medium. To take advantage of released hemoglobin, the hemoglobin assay kit (Sigma-

Aldrich) was used to measure lysis instead. A tendency for increased release of hemoglobin, possibly caused by killing of PRV-infected erythrocytes by cytotoxic cells, was observed (data not shown). However, differences between uninfected and infected individuals were not significant, and the variation within the control/infected groups were as high as the differences between the groups.

Despite the mentioned methodological challenges and partly excluded data, this trial lead to some noteworthy findings. We found that several cytotoxic genes were up-regulated in white blood cells isolated from spleen (figure 26 and 27) and we also found a larger population of CD8+ cells (figure 29) in PRV infected fish. It is likely that T- or NK-cell mediated killing of PRV-infected cells takes place in the infected salmon. The myocarditis seen in HSMI has been shown to be associated with influx of CD8+ cells. CD8α, CD8β and other genes involved in cytotoxic processes are up-regulated in hearts of PRV-infected fish (Johansen et al., 2015) correlating with decreasing levels of virus present. It is likely that CD8+ cells are involved in both the tissue damage characterized as HSMI and in controlling the infection. PRV also accumulates in the spleen after the initial infection of erythrocytes, and this could be caused by killing of infected erythrocytes here. At 7 wpc, increased expression of innate immune genes was correlated with high virus levels. Interestingly, at 8 wpc, increased expression of CD4 and CD8, and an increase in CD3+CD8+ cells were correlated with low virus levels. A logical explanation would be that high levels of virus stimulates the innate mechanisms more, and that the later adaptive response is important for clearance of the virus.

5 DISCUSSION

5.1 METHODOLOGICAL CONSIDERATIONS

5.1.1 Challenge trials

Challenge trials expose fish to stress and pain and should therefore not be performed without considering this cost against the value of the potential knowledge gained. Three R's (Russell & Burch, 1959) should be implemented in all experiments using animals with the aim of **R**educing the number of animals used, **R**eplace the use of animals with alternative methods and **R**efine the experiment, i.e. optimizing welfare by minimizing stress and pain, and improve study design to optimize the number of animals needed. It is widely accepted that fish can feel pain, fear and discomfort (Brown, 2015, Deakin *et al.*, 2019, Braithwaite & Ebbesson, 2014). Even though fish are protected by animal welfare legislations in the same way mammals are, the application of the 3Rs in aquatic medicine research and aquaculture lags behind when compared to what is in place in terrestrial farming and research on mammalian species.

All the plasma used in the papers included in this work have been "leftover" plasma from challenge trials performed for other purposes. Thus, they were perhaps not ideal for our purpose, for example challenge trials lasting longer that 15 weeks would have been desirable to determine long term antibody production. However, doing it this way reduced the number of challenge trials and fish used. It is a good option to obtain plasma from already executed or planned challenge trials if available and suitable. For the neutralization assays we attempted to do, blood had to be harvested specifically for this purpose. This was necessary as there is no cell line available that can support cultivation of PRV. We also did a small PRV i.p. injection challenge for the purpose of studying cytotoxicity. All animal experiments were approved by the Norwegian Animal Research Authority and performed in accordance with the recommendations of the current Norwegian animal welfare regulations.

For xMAP analysis of plasma, non-lethal sampling could have been an alternative, as only a few microliters are necessary for analysis. Non-lethal sampling has been performed in salmonid fish (Monte *et al.*, 2016, Collet *et al.*, 2015, Ye *et al.*, 2011b), and repeated anesthesia of pre-smolt Atlantic salmon does not seem to induce inflammation or stress (Chance *et al.*, 2018). By sampling from the same individual at every time point a reduction in animals needed would be achieved. It would also explain more of the host-pathogen interactions and time course of the immune reaction.

Challenge trials are performed in controlled environments on otherwise healthy individuals. They are well suited to detect effects of one factor, but the experimental conditions are different from the conditions in the field. This should be taken into account when evaluating results from challenge trials. We have seen differences in antibody binding to control proteins in challenge trials and in field studies. In paper III, supernatant from uninfected CHSE cells were coated on beads as a background control. No binding to these control beads was observed in the challenge trial, whereas high level of binding was observed from field samples. A likely cause of this is increased presence of polyreactive antibodies produced after infection with PRV or other unknown pathogens or after vaccination.

One must also be aware of the difference between cohabitation challenges and injection challenges. The natural infection route is through mucosal surfaces, and many of the mucosal immune mechanisms are bypassed when injecting the pathogen i.p. A study comparing expression of immune genes after i.p.- injection and cohabitation challenges with *Yersinia ruckeri* has demonstrated different expression patterns between the groups (Monte et al., 2016).

5.1.2 Monoclonal antibodies

Generally, the research on fish immunology is hampered by the lack of good monoclonal antibodies against key immunological markers. Most antibodies used in salmonid research are made against rainbow trout antigens. One example of a rainbow trout antibody used widely in Atlantic salmon research is the monoclonal mouse anti-rainbow trout IgM antibody "Mab4C10". In rainbow trout, this antibody recognizes both IgM-A and IgM-B. The original paper (Thuvander *et al.*, 1990) describing how this antibody was developed has been cited 125 times (Web of Science). It was later found that the antibody only recognizes the IgM-A isotype in Atlantic salmon (and only IgM-B in brown trout) (Kamil *et al.*, 2011, Kamil et al., 2013b). A study assessing several monoclonal antibodies, including Mab4C10, and one polyclonal antibody, concluded that the most commonly used antibodies against Atlantic salmon IgM only recognize a fraction of IgM-positive cells, ranging from 5 % to above 80 %. Only one of the three commercially available antibodies tested, the anti-salmonid IgH from Cedarlane, could recognize both isotypes (Hedfors et al., 2012). This is the antibody used in this PhD work. Previous studies describing antibody response or lymphocytes in Atlantic salmon can therefore unknowingly have used suboptimal antibodies or antibodies that only recognize one of the IgM isotypes. This is interesting when we know that the IgM-B of Atlantic salmon is reported to have a higher degree of disulfide bonding than IgM-A (Kamil, Raae et al. 2013) and that the degree of disulfide bounding between the monomers correlates with increased antibody affinity

and perhaps different effector functions (Ye, Bromage et al. 2010, Ye, Bromage et al. 2011, Ye, Kaattari et al. 2013).

In general, commercially available antibodies should be critically evaluated before being used as a research tool. A study from 2008 evaluated 6000 routinely used commercially available antibodies and found that less than half recognized only their specific target. Some producers tended to offer better antibodies, whereas others tended to offer consistently poorer ones (Berglund *et al.*, 2008).

5.1.3 Bead-based immunoassays

The xMAP assay is a valuable research tool for simultaneous detection of both specific antibodies to multiple antigens and of polyreactive antibodies after infection or vaccination.

There are no ELISAs or other serological tests against PRV available to compare our assay with, but in general the bead-based assay has several advantages compared to ELISAs, including lower cost, higher sensitivity, broader dynamic range and usage of smaller amounts of both sample material and detection antigen. The ambient analyte theory states that if using microscopic areas with a high concentration of capture molecules, the results are independent of both the concentration of the capture molecule and the sample volume. This is an important advantage compared to ELISAs where small differences in sample volume or amount of antigen attached to the wells can affect the result.

According to the coating manual (Bio-Plex Pro™ Magnetic COOH Beads Bio-Plex® COOH Beads Amine Coupling Kit Instruction Manual. Bulletin #4110012C), the optimal amount of capture protein on the xMAP beads will vary depending on the individual protein and should be titrated if possible. Seemingly paradoxical, the sensitivity can sometimes be increased by decreasing the amount of antigen coupled to the beads, with an improved linearity at lower concentrations. What we found was that 12 µg used for coating of beads gave slightly more linear titration curves than 6 µg or 24 µg when tested with recombinant PRV-1 µ1c. Ideally, we should have performed more titration testing, but unfortunately, we had limited access to antigen and sample material. This said, an implication of the ambient analyte theory is that the results are independent of the concentration of the immobilized capture molecule. This is in line with the results from testing different sample volumes and multiple incubations. The same was also found when testing different amounts of some of the other proteins in the conjugation reaction when coating the beads. The ambient analyte theory described for this type of immunoassays is obviously valid within certain limits. When adsorbing plasma (paper II), 160x the normal number of beads and six times the incubation time was used. This

led to a reduction of the binding to PRV σ1-coated beads from the PRV σ1-adsorbed samples to roughly half of the binding found from untreated samples.

Different dilutions of plasma were tested to find the concentration that best separated between positive and negative samples. Plasma from the PRV trials were diluted 1:50, 1:100, 1:200 and 1:400. The 1:100 dilution worked best with µ1c and µNS. For PRV1-σ1-LM, a lot of the samples had an MFI of around 25000, meaning that the amount of antibody is at or above the upper limit of quantification. Therefore, the samples should have been more diluted if we wanted to define the upper antibody level during infection. The 1:100 dilution was used with a goal to apply PRV1-σ1-LM and µ1c together in a multiplex assay. It would, however, have been interesting to see the antibody kinetics in more diluted samples.

When looking at the adsorption results from paper II, the background binding to irrelevant antigens is much lower than in the samples from the challenge trial, even on un-adsorbed plasma. The main difference between these analyses was that plasma from several fish were pooled for the adsorption and that a 1:400 dilution was used instead of the 1:100 dilution used for analysis of the challenge trial samples. The 1:400 dilution might be a better dilution for discrimination between positive and negative samples. From the equation mentioned in the immunoassay part,

$$\frac{[AbAg]}{[Ab][Ag]} = K_{eq}$$

follows that the higher the affinity (higher K_{eq}), the larger is the part of all the antibodies present that will be bound to antigen. Low affinity or non-specific antibodies can be present at a much higher concentration than specific antibodies and still bind less antigen. We can use this principle in immunoassays to dilute samples until such a small part of antibodies with low specificity bind antigen that it is below the limit of detection or below the "background" in the assay. A 1:200 dilution had been previously tested (data not shown), but without decreasing the difference between specific binding and background. This principle also works the other way around. High concentrations of low affinity antibodies will still bind so some extent if the concentration is high enough. It is therefore likely that the unspecific binding we see is caused by very high levels of non-specific antibodies binding to antigens with low affinity. Since the plasma was pooled, it is also possible that some of the non-specific antibodies bound to proteins in plasma from other fish before the beads were added.

Another difference in conditions when analyzing samples is also interesting to note. The xMAP analysis on the pre-adsorbed plasma was performed in May 2018, an especially warm month with temperatures in the lab reaching up to around 30°C (normally between 20 and 25°C). From the laws

of thermodynamics follows that a chemical reaction will only happen spontaneously if the change in Gibbs energy is negative. The change in Gibbs energy, ΔG, is dependent on changes in enthalpy and entropy. The ΔG for the antibody binding to an antigen (association) is ΔG_{ass}.

$$\Delta G_{ass} = \Delta H_{ass} - T\Delta S_{ass}$$

where H is enthalpy, T is the absolute temperature and S is entropy. ΔG must be negative for the reaction to occur. An exothermic reaction is a reaction where ΔH is negative, and this is the case with formation of antigen-antibody complexes. For a polyreactive, flexible antibody to bind with an antigen, it will have to change its conformation. The conformation change is not spontaneous, as it decreases the entropy of the system, meaning that ΔS will be negative. The last part of the equation will then be positive, and ΔG will only be negative (the reaction will occur) if the absolute value of TΔS is smaller than the absolute value of ΔH. In other words, the reaction will be more skewed towards unbound antibodies if the temperature is high. A high affinity, highly specific antibody working as the "lock and key" analogy will not change its conformation to bind antigen, and ΔS will be around zero. TΔS will then not change much with changes in temperature. ΔH is still negative, and the reaction will occur, and might even be increased at higher temperatures. This is of course only speculations, but it has been described that for low specificity polyreactive IgM monomers, a temperature increase of only 10 degrees (from 25°C to 35°C) reduced the binding affinity with between 10-40-fold. For affinity maturated IgG antibodies, reduction of binding affinity was only 2-fold (Manivel et al., 2000). The ambient temperature also affects the time for the reaction to reach equilibrium, and this might, in practice, be more important for any temperature-dependent degree of antibody binding. This mechanism works in the opposite way, as equilibrium is reached faster with increasing temperatures.

In paper II, we found increasing levels of unspecific binding in PRV infected fish, likely caused by polyreactive antibodies. Heat treatment (48 °C for 20 minutes) reduced this unspecific binding in control fish and against control proteins, while the specific binding to PRVσ1 in plasma from PRV-infected fish remained high. When the temperature increases, proteins will denature. Denatured proteins change from their native folded state into an unfolded state. Unfolded proteins expose hydrophobic amino acid side chains, causing them to aggregate with other folded and unfolded proteins. This is driven by a process to minimize contact between hydrophobic residues and water molecules. The thermal stability of a protein is determined by the protein itself (primary, secondary and tertiary structure) and factors in the environment. The denaturation can be either reversible or irreversible. Each antibody domain denatures at a different temperature, and this denaturation is reversible. Mixes of folded and unfolded antibody domains will tend to make polyprotein aggregates. When the domains interact in these aggregates, the denaturation can become irreversible (Akazawa-

Ogawa *et al.*, 2018, Vermeer & Norde, 2000). Heavy chains tend to aggregate easier than light chains, and antibody isotypes denature at different temperatures. Of the mammalian isotypes, IgE is especially heat labile, and the Fc of IgE is denatured at 56 °C (Akazawa-Ogawa et al., 2018).

There are several possible explanations of why, at 48 °C, the binding by non-specific antibodies was reduced, and not the specific binding. The thermostability of an antibody is increased with more disulfide bridges. Introduction of disulfide bonds can stabilize proteins (Matsumura *et al.*, 1989, Trivedi *et al.*, 2009), and such bonds have been credited for giving native antibodies their stability (Goto & Hamaguchi, 1979, Frisch *et al.*, 1996). When an extra disulfide bond was added in the C_H2 of IgG, it seemed to also have an indirect stabilizing effect on the C_H3 and Fab domains as well (McConnell *et al.*, 2013). In this experiment the thermostability of the whole IgG molecule increased. It has been observed in single domain antibodies (from camelids) that the introduction of more disulfide bindings leads to an increase in both affinity and heat stability (Zabetakis *et al.*, 2014). Rabbit IgGs with more disulfide bonds between the variable and constant domain in the light chain were also more heat stabile, but the affinity was not affected (Kawade *et al.*, 2018). Increased numbers of disulfide bridges correlates with increased affinity in rainbow trout IgM (Ye et al., 2010) and the stability of Atlantic salmon IgM-B, which has the most disulfide bonds, was distinctly higher compared to IgM-A when subjected to extreme heating conditions (Kamil et al., 2013b). It is therefore likely that polyreactive or non-specific antibodies have fewer disulfide bridges than specific antibodies, and thus are more heat labile.

Another possible factor is that the antibody flexibility is affected by heat treatment. High affinity antibodies are considered to bind antigen in a “lock and key” fashion as the high numbers of binding interactions contribute to the rigidity of the paratope. In contrast to the rigid structure of this classic antibody model, it is believed that polyreactive antibodies have more flexible antigen binding sites and are able to change conformation to accommodate different antigens (Notkins, 2004). This is explained by fewer electrostatic contributions to the antigen binding and increased hydrophobicity, leading to greater molecular flexibility (Torres & Casadevall, 2008).

As polyreactive/natural antibodies are known to bind to self-antigens or damaged self (Casali & Notkins, 1989, Notkins, 2004, Merbl et al., 2007), it is also possible that the polyreactive antibodies in plasma binds to other plasma proteins denatured by the heat treatment. When proteins unfold, linear epitopes that are hidden when the protein is in its native form, can be bound by antibodies. This would decrease the numbers of polyreactive antibodies available for binding to antigen on the beads.

Thim et al (Thim *et al.*, 2014) showed reduced titers of neutralizing antibodies against SAV after heat treatment (56 °C for 30 min). They suggest a role of complement mediated neutralization against SAV even though addition of naïve serum as a complement source in the heat-treated groups did not increase the titers for the high titer groups. At this temperature and time, we have seen that the amount of detected antibodies was reduced to around half both in PRV-infected and control fish (supplementary figure S4 in paper II). This indicates that the reason for the reduced titers in this publication was not caused by complement inactivation, but perhaps by denaturation of antibodies. Proteins of cold-water fish like salmon must function at lower temperatures than their mammalian counterparts. It has been shown that proteins like collagen and myosin are less heat stable in cold water fish than in mammals (Somero, 1983, Poulter *et al.*, 1985, Chen *et al.*, 2004), and it is reasonable to assume that the same is true for antibodies.

5.2 General discussion

Circulating antibodies tell the story of what the salmon's immune system has encountered. Measuring antibodies can therefore not only tell us about current infections in the fish, but also about previous infections and vaccination status. To do this, we need good antigens that bind specific antibodies and a reliable method to measure this binding. Creating good serological assays for fish has been challenging due to background binding, possibly caused by polyreactive antibodies. ELISAs have been used, but high background titres and low specific responses is a common challenge. The present work has used a bead-based multiplex immunoassay to confirm that PRV-infected Atlantic salmon and rainbow trout produce specific antibodies against PRV proteins and that SAV2- and SAV3-infected Atlantic salmon produce specific antibodies against SAV particles. The antibody kinetics seem to be quite consistent, with a rise starting between the time of peak virus levels in blood and two weeks after this. For PRV, a maximum antibody level was found around 8-12 wpc coinciding with regeneration in the heart.

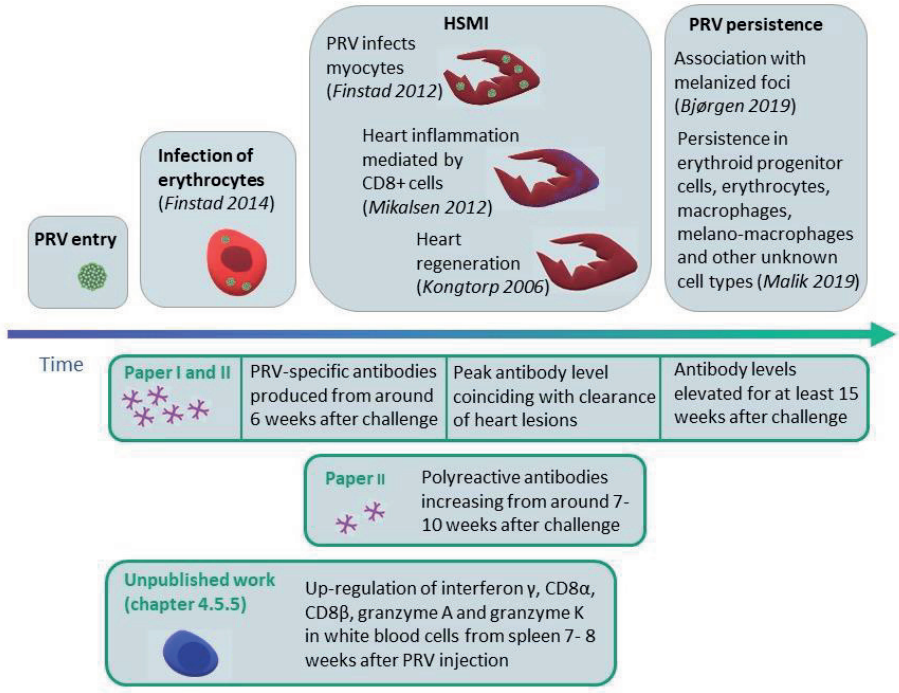

***Figure 32**. Summary of stages of a PRV infection including results from paper I, paper II and the QuantiGene Plex assay described under unpublished work.*

Figure 32 summarizes our results regarding PRV infection. In paper I and II, antibodies against two outer capsid proteins and one non-structural protein of PRV were detected. The specificity of the antibodies against the PRV-σ1 protein was evaluated in paper II, using heat-treatment and pre-adsorption of samples. This was not done in paper I where beads coated with PRV-1 proteins µ1c and µNS were used. Heat-treatment was, however, tested later, and in contrast to the irrelevant antigens used in paper II, this did not reduce the binding to µ1c and µNS. Further supporting specific binding to µ1c and µNS, and in contrast to σ1 and the irrelevant antigens in paper II, no background binding to µ1c or µNS was observed in plasma from control fish. PRV µ1c is a structural protein, and it expected to be a target for antibodies in PRV-infected fish. On the other hand, µNS is a non-structural protein, and only present intracellularly during viral replication. Antibodies against non-structural proteins are, however, detected after infection with other viruses, like poliovirus (Ehrenfeld *et al.*, 1995), foot-and-mouth disease virus (Mackay *et al.*, 1998), dengue virus (Valdés *et al.*, 2000) and Zika virus (Gao *et al.*, 2018). Immunization with non-structural proteins or injection with monoclonal antibodies against non-structural proteins can also protect against infection with several flaviviruses (Chung *et al.*, 2006). Virus-infected cells, and killed or lysed cells in particular, can be phagocytized by APCs, and cytosolic viral proteins can then be presented on MHC-II. Cytosolic protein may also, in some cases, be presented by MHC-II instead of MHC-I on APCs, for example by the autophagy pathway (Abbas et al., 2015). It is therefore not unexpected to find antibodies against µNS.

We also found cross-reactivity between antibodies targeting different PRV subtypes (paper II), where antibodies from PRV-3-infected rainbow trout could bind to beads coated with PRV-1 σ1. An assay using σ1 seems to be better suited to detect anti-PRV-3 antibodies than assays using µ1c or µNS. Only one PRV-3-infected rainbow trout from paper II showed any binding to PRV-1 µ1c-coated beads, and PRV-3-infected rainbow trout plasma showed low binding to µ1c beads in another publication (Vendramin et al., 2019). PRV-3 µ1 showed only one band on western blot when rabbit antisera raised against PRV-1 µ1 was used as primary antibody (Dhamotharan *et al.*, 2018). In contrast, µ1 from PRV-1 showed three bands. The two µ1 proteins have a 91,5 % amino acid similarity, but the location of the putative cleavage site between µ1n and µ1c is different. This could be an explanation for the lower antibody binding across PRV subtypes for the µ1c antigen. An assay multiplexing σ1 and µ1c could possibly be used to differentiate between populations infected with PRV-1 and PRV-3. However, the viruses mainly cause disease in separate species, so the practical use of a discriminating assay is uncertain.

We saw, also in paper II, that a PRV infection leads to an increase in polyreactive or polyclonal non-specific antibodies, and a background antibody binding in healthy fish to both lipid-modified PRV-σ1

and irrelevant proteins, which was reduced by heat inactivation (paper II). We have also seen a slight increase in antibody binding to irrelevant protein after SAV challenge, but much less pronounced than the increase seen in PRV challenges. In mammals, a polyclonal B cell activation with production of non-specific antibodies can be observed after infection with many types of pathogens (Montes *et al.*, 1999, Reina-San-Martin *et al.*, 2000). This B cell activation can be independent of T cell help (Montes et al., 2007), and the B cells can be either B1 or B2 (Julien *et al.*, 2002). These antibodies may represent a critical part of the early defense against pathogens, and may also neutralize virus (Klasse, 2014). Controversially, it has also been suggested that the polyclonal antibody production can help pathogens avoid the immune system, as the antibodies produced are not specific for the pathogen or by enhancing uptake of virus by phagocytosis (Montes et al., 2007). They may also contribute to autoimmune manifestation of chronic disease as the polyreactive response could turn into an anti-self response (Montes et al., 2007). It is not clear what effect the increase in these non-specific antibodies has on the pathogenesis of HSMI or other manifestations of PRV-infections.

In paper III, we detected antibodies against SAV. Both the recombinant SAV3 E2 protein and SAV3 particles disrupted with Triton-X worked well as antigens for both SAV2- and SAV3-infected fish in a challenge trial. However, when used on plasma from a field outbreak of PD caused by SAV2 and on plasma from PRV-infected (SAV free) fish, we found high levels of unspecific binding to E2. As the E2 used in the assay was a recombinant protein made in *E. coli*, it is not glycosylated and may not be folded and expressed in a manner similar to the native antigen. This is a likely reason for the aberrant binding properties in comparison to native virus. The binding observed was likely caused by a combination of polyreactive antibodies and specific antibodies against linear epitopes on E2. As we have shown in paper II, polyreactive antibodies are produced after PRV infection. They can also be produced after vaccination (Lund et al., 2019) and after exposure to the environment or other pathogens (Kachamakova *et al.*, 2006). The fish from the PD outbreak had previously been infected with PRV and were vaccinated against IPNV and several bacterial diseases. Despite of this, disrupted SAV particles seemed to predominately bind SAV-specific antibodies. Beads coated with these disrupted particles did not bind any antibodies in plasma from PRV-infected fish.

In United Kingdom and Ireland, a neutralization test for SAV is in use for diagnostics, but in Norway only detection of viral RNA by RT-qPCR on heart is used routinely today. An xMAP assay with disrupted SAV particles could be used in diagnostic of SAV as a complement to PCR methods, especially since non-lethal sampling is possible and as the xMAP assay only takes one day compared to at least three days for the neutralization assays. Antibody detection can be used to detect fish and fish populations that have been previously infected and have cleared the pathogen, when the pathogen is located in tissues not sampled, or in subclinical infections. Serology with a neutralization

assay was shown to detect more infected fish than RT-qPCR in a subclinical outbreak of SAV1 (Graham et al., 2006). We also saw this in paper III, where 70% of samples were negative for viral RNA 13 and 15 weeks after the PD outbreak while most samples had high levels of antibodies, both when measured with the neutralization assay and with the xMAP assay. According to Norwegian regulations, a verified PD outbreak requires that at least two independent laboratory tests have detected SAV or antibodies against SAV or histopathological findings consistent with PD in the same. RT-qPCR of hearts and histopathology is routinely used. Virus can be found long before pathological changes can be detected. This means that SAV-infected fish can release virus for an extended period of time without being treated as a PD outbreak, increasing the risk of spreading virus to other locations. If serology was used instead of histopathology, measures to prevent the spread of the virus could be applied much faster. The assay should also be tested on fish vaccinated against PD. Inactivated vaccines containing complete viral particles could potentially preclude the use of serology for diagnostics in vaccinated populations. Subtype vaccines or DNA vaccines can be designed to facilitate serological differentiation between vaccinated and infected animals.

The antibodies against SAV, detected in the xMAP assay, were strongly correlated (Spearman's ρ = 0,73 ($p<0,0001$)) with titers from the virus neutralization assay. There were, however, fish with very high values measured with the xMAP method that had very low titers or were negative for neutralizing antibodies and vice versa. This indicates that SAV-specific antibodies and SAV-neutralizing antibodies are different but partly overlapping variables and that not all fish manage to produce antibodies against the neutralizing epitopes. This also indicates that only some of the antibodies binding to SAV are neutralizing and that some of the neutralizing antibodies does not bind strongly to the E1, E2 or cap proteins. A study by Chang et al, published in 2017, compared protection and presence of neutralizing antibodies against SAV in fish vaccinated with a commercially available inactivated vaccine, a DNA vaccine based on the complete SAV structural polyprotein and a DNA vaccine containing only the E2 protein. The commercial vaccine and the DNA polyprotein vaccine reduced the virus level in serum, but only the latter protected against pathological changes. In addition, only fish vaccinated with the DNA polyprotein vaccine had increased levels of antibodies against E2 measured with ELISA and increased neutralizing activity against SAV3. The authors suggest that the protection is in part mediated by the neutralizing antibodies (Chang et al., 2017). In a co-infection study with SAV and PRV, plasma from PRV-infected fish was shown to neutralize SAV (Rosaeg et al., 2017). The neutralizing effect was lost in the majority of samples after heat treatment (48°C for 20 minutes). Unless the neutralization was caused by other, unknown factors in plasma, this suggests that heat labile, non-specific and possibly polyreactive antibodies can have a neutralizing effect on virus, either alone or in a complement-dependent manner. Non-neutralizing

antibodies may bind to any available epitope on a virus. In contrast, neutralizing antibodies likely bind to a limited number of specific, possibly conformation-dependent, epitopes. Therefore, although less likely than with specific antibodies, polyreactive antibodies can be neutralizing if binding to the right epitope. The relevance of the neutralizing and the specific antibodies in relation to protection against PD requires further research.

We did not manage to detect neutralizing antibodies towards PRV, as PRV did not stably infect erythrocytes ex vivo. In mice, monoclonal antibodies against several MRV proteins are shown to be neutralizing and can inhibit infection of cell cultures and systemic lethal reovirus infection (Tyler et al., 1993, Helander *et al.*, 2004, Hutchings *et al.*, 2004). If neutralizing antibodies against PRV are produced by infected fish, a cell line where PRV can be propagated is needed to detect this.

An important motivation for studying antibody production and functions in Atlantic salmon is to provide a basis for vaccine development and evaluation. Serology has been suggested as the best available correlate for protection after vaccination (Munang'andu & Evensen, 2019). This seems to be true for some viruses, but not for all. The use of antibody levels as a correlate does not necessarily mean that the protection is mediated by the antibodies, but the level of antibodies could correlate with other long term protective immune responses. We have measured IgM in blood, but especially IgT, and perhaps also IgD, in the mucosa could be of greater importance for protection, especially since viruses naturally infect via mucosal surfaces.

Many vaccine trials are performed with just a few weeks between vaccination and challenge, like for example 6 weeks in two PRV trials (Haatveit et al., 2018, Wessel et al., 2018a), 10 weeks in a SAV trial (Chang et al., 2017), 4 and 8 weeks in a *Yersinia ruckeri* trial (Raida & Buchmann, 2008) and 6 weeks in a ISAV trial (Wolf et al., 2013). Considering our findings that specific antibodies start to develop after around 6 weeks after cohabitation challenge, it appears that these challenges are either performed at the peak level of antibody production or too early for adaptive immune responses to develop. The potential protection is therefore mediated either by the primary antibody response caused by the vaccine, by polyreactive antibody responses or by other parts of the immune system. Innate IFN-regulated responses can last for up to 11 weeks after i.p. injection of PRV (Johansen et al., 2015, Dahle et al., 2015), and is a possible cause of protection in a subsequent challenge. Polyreactive antibodies could also affect vaccine efficacy. In goldfish, high levels of pre-existing non-specific antibodies was correlated with better protection against *Aeromonas salmonicida*, but also with no significant production of specific antibodies following vaccination (Sinyakov et al., 2002). This should be studied in Atlantic salmon.

Any protection against PRV or SAV infection, or the influence on HSMI or PD pathology by specific, neutralizing, non-neutralizing or polyreactive antibodies remain to be determined. The ability to induce antiviral innate immune responses seems to be coupled with the ability to cause more severe disease when it comes to PRV (Wessel et al., 2018b). We also found that interferon-γ and Mx gene expression levels correlated with the highest virus levels in blood seven weeks after i.p. injection with PRV. Interestingly, we also found an up-regulation of genes encoding both CD8 and CD4 in spleen, correlating with lower virus levels in blood one week later. The HSMI pathology is reduced after the maximum levels of specific antibodies and virus levels in heart decline after CD8+ cells are recruited there (Finstad et al., 2012). This points to a role of both arms of the adaptive immune system in clearing HSMI lesions in the heart. Still, PRV can escape the immune system and cause a persistent infection.

The possibility of multiplexing is perhaps the greatest advantage of the xMAP method. Antigens from several pathogens could be used together to make a diagnostic assay for the aquaculture industry. This is, however, dependent on finding good antigens that antibodies can bind specifically with limited unspecific binding. The high levels of unspecific binding could be an important limitation of a serological assay in aquaculture, especially when used in field settings. All of the antigens we have used in our xMAP assays and the neutralization assay likely detect both non-specific and specific antibodies to a certain extent. The degree of specific versus non-specific is, however, very different. Both SAV-TX and PRV-µ1c seem to bind almost exclusively specific antibodies, whereas lipid modified PRV-σ1 and SAV-E2 also bind many polyreactive antibodies. Thorough testing in infected as well as vaccinated fish, with both heat-treatment of samples and pre-adsorption, should be performed when evaluating new antigens.

6 MAIN CONCLUSIONS

- PRV-1-infected Atlantic salmon produce specific antibodies directed toward the PRV-1 proteins µ1c and µNS. PRV-1-infected Atlantic salmon and PRV-3-infected rainbow trout produce specific antibodies against the PRV-1 protein σ1.
- A PRV-1 infection induces production of high levels of non-specific or polyreactive antibodies.
- SAV-infected fish produce SAV-specific antibodies and neutralizing antibodies, and the levels of these are strongly correlated.
- The bead-based immunoassay described in this thesis has great potential for use in salmonids but require good antigens and control of unspecific binding.

7 FUTURE PERSPECTIVES

Looking at the dynamics of polyreactive and specific antibodies in the same fish throughout the course of an infection or after vaccination would be very interesting. As the bead-based immunoassay requires only minute amounts of sample material, it opens up the possibility of non-lethal sampling of blood or mucus, and the chance to follow the changes in antibody levels in the same fish over time. This could reveal more information about the host-pathogen interactions and potential correlations between antibodies and protection. In SAV-infected fish, non-lethal sampling of plasma could be used for both xMAP and neutralization assays. This could be used to study the humoral response throughout an infection or after vaccination and the effects on the outcome. It would also be intriguing to look for mucosal antibodies. The same method used for plasma could be used to detect IgM in mucus or mucosal tissues. IgT can be detected when an anti-IgT antibody that recognizes Atlantic salmon IgT is available. Antibody levels should also be monitored for a longer period than the 10-15 weeks studied in this work.

I.p. challenges are used for testing the safety and potency of every vaccine batch produced, and the majority of animals used in research in Norway are fish used for this purpose. Sub-potent vaccine batches could instead be identified by detecting antibody levels. After a furunculosis vaccine, the antibody level was shown to be dose-dependent and correlated with the amount of antigen in the vaccine and also with protection (Romstad *et al.*, 2013). This was done with ELISA, and the conclusion was that antibody measurement was better at identifying sub-potent vaccine batches than testing protection by i.p. challenges. With the 3Rs in mind, antibody detection by bead-based immunoassays would be a better method for batch potency testing of vaccines in the future.

The bead-based immunoassay we have used is suitable for simultaneous detection of antibodies against several pathogens. Antibodies remain in the blood after infections are cleared and can be detected by serology even when no pathogen is present to be detected. A multiplex immunoassay with antigens from several pathogens could therefore be a valuable tool for screening and for diagnostic purposes in aquaculture. The assay could also be used in other domestic animals where ELISAs are used today. Proper validation must be done in order to make assays for diagnostic use. A validated multiplex immunoassay detecting antibodies against the most common pathogens in aquaculture could contribute to faster diagnosis, better surveillance and improved control of infectious diseases. This would in turn decrease the loss of fish and contribute to improved welfare and sustainability.

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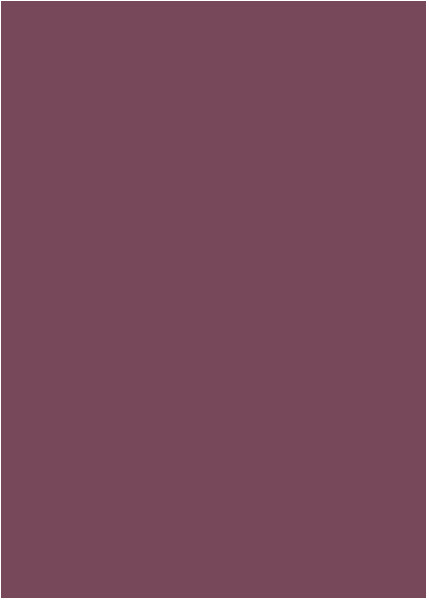
APPENDIX: ENCLOSED PAPERS I-III

Paper I

I



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Full length article

A bead based multiplex immunoassay detects *Piscine orthoreovirus* specific antibodies in Atlantic salmon (*Salmo salar*)

Lena Hammerlund Teige [a], Morten Lund [b], Hanne M. Haatveit [a], Magnus Vikan Røsæg [a, c], Øystein Wessel [a], Maria K. Dahle [b, *], Anne K. Storset [a]

[a] Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, N-0454 Oslo, Norway
[b] Norwegian Veterinary Institute, N-0454 Oslo, Norway
[c] SalMar ASA, N-7266 Kverva, Norway

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ABSTRACT

Future growth in aquaculture relies strongly on the control of diseases and pathogens. Vaccination has been a successful strategy for obtaining control of bacterial diseases in fish, but for viral diseases, vaccine development has been more challenging. Effective long-term protection against viral infections is not yet fully understood for fish, and in addition, optimal tools to monitor adaptive immunity are limited. Assays that can detect specific antibodies produced in response to viral infection in fish are still in their early development.

Multiplex bead based assays have many advantages over traditional assays, since they are more sensitive and allow detection of multiple antigen-specific antibodies simultaneously in very small amounts of plasma or serum.

In the present study, a bead based assay have been developed for detection of plasma IgM directed against *Piscine orthoreovirus* (PRV), the virus associated with the disease Heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon. Using recombinant PRV proteins coated on beads, antibodies targeting the structural outer capsid protein μ1 and the non-structural protein μNS were detected. Results from a PRV cohabitation challenge trial indicated that the antibody production was initiated approximately two weeks after the peak phase of PRV infection, coinciding with typical HSMI pathology. Thereafter, the antibody production increased while the epicardial inflammation became less prominent.

In conclusion, the novel assay can detect PRV-specific antibodies that may play a role in viral defence. The bead-based immunoassay represents a valuable tool for studies on HSMI and possibly other diseases in aquaculture.

1. Introduction

Assays detecting circulating antibodies specific for pathogenic microbes have been used for centuries in mammals to diagnose previous exposure to disease. The isotype of specific immunoglobulins (IgM, IgG) as well as increased titres of specific, high affinity antibodies during the first weeks after infection provides valuable diagnostic information. The situation in salmonid fish is slightly different, and serologic tests have not been widely used to define infection status in aquaculture. As IgM is the dominating isotype in serum of salmonids and no isotype switch is evident, the presence of IgM does not necessarily imply recent infection. As fish IgM in general is of lower specificity than mammalian IgG, there is an increased risk of detecting low affinity cross-reactive antibodies in non-infected fish and hence, to have false positive results. However, studies in rainbow trout (*Oncorhynchus mykiss*) have shown that although low affinity IgM is present early after infection, high affinity IgM develops later during the immune response [1,2].

Reports on detection of specific antibodies against viruses or viral vaccine antigens in Atlantic salmon is limited and have

* Corresponding author. Norwegian Veterinary Institute, Ullevalsveien 64, N-0454 Oslo, Norway.
E-mail address: maria.dahle@vetinst.no (M.K. Dahle).

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primarily been based on traditional Enzyme-Linked Immunosorbent Assays (ELISAs) with whole viral particles as capture antigen [3]. For infectious salmon anemia virus (ISAV) and viral hemorrhagic septicemia virus (VHSV) which both cause disease in salmonids, vaccines have been developed based on recombinant viral proteins, and traditional ELISAs based on these antigens have been used for antigen-specific antibody detection [4–6]. For detection of specific antibodies for other viral infections, neutralizing assays, western blot assays and indirect immunofluorescence antibody assays have been used [7–9]. However, these methods are time consuming and require large sample volumes. Furthermore, for viral diseases where the virus cannot be grown in cell culture, neutralization assays are no option and the alternatives are even more limited.

Bead based multiplex immunoassays have been successfully used to detect mammalian antibodies for more than a decade [10–12]. The assay system is based on up to 100 separate bead populations defined by fluorescent dyes that can be analysed in one single well. The beads can easily be covalently coupled with antigen and can be used to detect specific antibodies in serum or tissue fluids. This enables detection of antibodies targeting a wide range of antigens as well as controlling unspecific background in one single well. Compared to traditional ELISAs, the bead-based assays have an increased dynamic range, are labour and time saving, gives lower background and require only small amounts of antigen for coating and a small volume of sample material. It has also been shown that the bead based assays have higher sensitivity compared to ELISA [10].

Heart and skeletal muscle inflammation (HSMI) is a common disease in Norwegian aquaculture [13]. Although HSMI mortality is often moderate, the disease is prevalent enough to be considered one of the main viral disease problems in Norwegian farmed salmon. More recently, HSMI cases have been identified also in Chile and Canada [14,15], observations that are causing concern and increasing international focus on the disease. HSMI is characterized by epicarditis, panmyocarditis and inflammation in red skeletal muscle [16]. *Piscine orthoreovirus* (PRV) was described to be associated with HSMI in 2010 [17], and found to be ubiquitously distributed in farmed Atlantic salmon in Northern Europe, Chile and North America [18–20].

PRV is a nonenveloped, icosahedral dsRNA-virus. Its genome consists of 10 segments that encode at least eight structural and two non-structural proteins [17]. Salmon myocytes, erythrocytes and macrophages are target cells for PRV [21–23], and the infected cells contain virus localized in cytoplasmic inclusions described as viral factories [21]. The non-structural PRV protein μNS is responsible for the formation of viral factories and the assembly of other viral proteins [24]. As further information about the functions of PRV proteins is not available, the mammalian orthoreovirus (MRV) can be used as a model for predicting functional properties [25,26]. The MRV proteins σ1, σ3, μ1 and λ2 are components of the outer virus capsid, and their analogues have been identified in PRV [27]. MRV attaches to target cells by binding of the σ1 protein to surface receptors which triggers internalization of the virus by receptor mediated endocytosis [28]. The membrane penetration of the virus particle is dependent on μ1, which is proteolytic cleaved into μ1c and a short μ1n peptide [29]. Antibodies recognizing σ1, σ3, μ1c, and λ2 can inhibit MRV infection of cell cultures as well as systemic lethal reovirus infection in mice [30–32]. It is thus likely that σ and μ protein analogues in PRV are targets for antibodies in infected salmon and that neutralizing antibodies may have a role in defence against the virus.

The aim of this study was to establish a bead based multiplex immunoassay based on recombinant proteins for the detection of PRV-specific antibodies in Atlantic salmon.

2. Materials and methods

2.1. Experimental fish, infection and sample collection

Atlantic salmon (*Salmo salar*) parr (N = 500, mean weight of 42.5 g (range 27.3–59.1 g), mixed gender) of a SalmoBreed strain (Bergen, Norway) were transferred from the VESO hatchery to VESO Vikan aquatic research facility (Namsos, Norway). The experimental fish were unvaccinated and confirmed negative for common bacterial and viral infections. In particular, the presence of PRV was assessed by RT-qPCR and confirmed absent in heart, spleen and blood in 14 fish before initiating the experiment, and from eight fish from a negative control group at all time points sampled. The challenge trial was performed on fish undergoing controlled light-induced smoltification initiated by six weeks of 12 h daylight and 12 h darkness (12/12 L/D) followed by six weeks of constant daylight (24/0 L/D). The experiment was run in fresh water at 12 °C (±1 °C). The fish were fed 1% of total biomass per day throughout the challenge trial and were starved 24 h before sampling. The fish were challenged with PRV two weeks after initiating the smoltification regime of 12/12 L/D. The PRV challenge was performed by i.p. injecting naïve fish (N = 164) with 0,1 ml of a PRV positive inoculum consisting of pelleted blood cells collected in a previous cohabitation trial (VESO Vikan), i.e. the second passage in experimental fish. This material is routinely used for PRV challenge studies at VESO Vikan and the PRV RNA level in the blood pellet was determined to a Ct value of 17,3 by RT-qPCR using 100 ng total RNA input and diluted sixfold prior to inoculation. The material originated from a Norwegian field outbreak of HSMI in 2012. After removal of the adipose fin, the injected fish were randomly divided into each of two replicate fibreglass tanks (1.5 m^3) containing N = 82 naïve cohabitants in a 1:1 (shedder:cohabitant) ratio. The control group consisted of N = 164 naïve fish. The challenge trial lasted for 10 weeks post PRV challenge (WPC), and the fish were monitored on a daily basis. The experiment was approved by the Norwegian Animal Research Association and followed the European Union Directive 2010/63/EU for animal experiments.

2.2. Sample collection

The fish allocated for sampling (8 cohabitant fish or controls per tank) were euthanized by bath immersion using benzocaine chloride (1 mg/5 L water) (Apotekproduksjon AS, Oslo, Norway) for 5 min in a separate container. Organ samples from heart, spleen and heparinized blood were collected 4, 6, 8 and 10 WPC. Samples were collected from N = 8 fish per group at each time point and preserved on RNA*later*™ and phosphate buffered 10% formalin for RT-qPCR analysis and histopathological evaluation, respectively. Heparinized blood was collected from the caudal vein. Ten fish sampled before PRV challenge served as negative controls. All samples were stored cool until shipment to the Norwegian Veterinary Institute (NVI). Following arrival at the NVI laboratory, samples on RNA*later*™ and a sub-sample of 200 μl heparinized blood were shipped cooled to PatoGen AS (Ålesund, Norway) for RT-qPCR analysis. The rest of the blood samples were transferred to an empty 1,5 ml Eppendorf tube and centrifuged at 3000 rpm for 10 min at 4 °C. Plasma was collected and stored at −80 °C until further analysis. Tissue samples on phosphate buffered 10% formalin were transfer to 70% ethanol 36 h after sampling and stored at 4 °C.

2.3. RT-qPCR

RNA extraction and RT-qPCR analysis for PRV RNA in heart, spleen and heparinized blood was performed by PatoGen AS. The

PRV assay used is described by Glover et al. [33], and validated to ISO17025 standards [33]. An RT-qPCR assay for elongation factor 1α (EF1α) was performed as described earlier [34], and used for normalization (Ct^{PRV} - $Ct^{EF1\alpha}$). The cut-off Ct value (i.e. no target RNA detected) was set to 37.0.

2.4. Histopathology

Formalin-fixed hearts sampled at 6, 8 and 10 WPC were stored at 4 °C until standard dehydration and paraffin wax embedding. Sections of 4–6 μm were stained with hematoxylin and eosin according to standard procedures. The samples were randomized and blind scoring was performed. An ordinal scoring system for epicarditis and myocardial inflammation ranging from no pathological changes (0) to mild (1), moderate (2) and extensive (3) was used. The scoring criteria were adapted to the range of inflammation in the samples after an initial screening, and is displayed in Supplementary Table S1.

2.5. Recombinant PRV-proteins

The PRV proteins σ1, σ3 and μ1c were produced as his–tagged recombinant proteins in *E.coli* (BL21 Star DE3 strain, Invitrogen, Carlsbad, USA) and purified on a nickel column. The details of the expression and purification of the recombinant PRV σ1 [22], σ3 [27] and μ1C [22] proteins have been previously described.

A lysate based on untransfected *E. coli* was prepared to serve as background control for the recombinant PRV σ1, σ3 and μ1C proteins described above. Briefly, untransfected *E. coli* (BL21 DE strain, Invitrogen) was grown overnight in LB medium and centrifuged at 3000 g for 10 min at 4 °C. The pellet was frozen and thawed and suspended in ice-cold phosphate buffered saline (PBS). The solution was sonicated on ice for 10 s five times with 30 s rest in between. The sample was passed through a 21G syringe three times, sonicated and filtered through a 0.45 μm filter (Corninge Incorporated, Corning, USA).The filtered lysate was diluted in PBS+0,5% BSA to make the protein concentration similar to that of the μ1c sample. Protein content was measured with BSA and γ-globulin as standard reference (Protein Assay, Bio-Rad Hercules, CA, USA), on NanoDrop (Saveen Werner, Limhamn, Sweden), and with a 650 nm filter on Titertek multiskan PLUS (Labsystems, Finland).

The μNS protein was produced using the BaculoDirect™ Baculovirus Expression System (Invitrogen) following the supplier's protocols. For construction of the Gateway® entry vector, sequence of the μNS open reading frame (ORF) was obtained by PCR amplification using the forward primer 5′-CACCATGGCTGAATCAATTACTTTTGGAGGA-3′ (startcodon underlined), the reverse primer 5′-GCCACGTAGCACATTATTCACGCCCAC-3′ and plasmid pcDNA3.1 μNS N-FLAG [24] as template. The PCR product was cloned into the Not1 and Asc1 recognition site of the pENTR™ TOPO® vector (Invitrogen) and the pENTR μNS construct was used to perform a LR recombination reaction and generate the corresponding recombinant baculovirus DNA. Spodoptera frugiperda (Sf9) insect cells (BD Bioscience, Erembodegem, Belgium) cultured in Grace Insect Medium (Invitrogen) was transfected with recombinant baculovirus DNA according to supplier's protocol and P1 viral stock was harvested 11 days post transfection. A Western blot analysis was performed to verify expression of the recombinant protein. The P1 viral stock served to produce the next high titer viral stocks according to supplier's protocol. The BacPAK qPCR Titration kit (Clontech, Mountain View, USA) was used to determine viral titre. Finally, Sf9 insect cells were infected with P2 or higher passage recombinant baculovirus stock (>1×10^8 copies/mL) and incubated at 27 °C for 96 h for expression of the recombinant μNS C-terminal His-tagged fusion protein.

The cells were pelleted by centrifugation for 10 min at 5000 rpm, resuspended and washed in PBS. Purification of recombinant protein was carried out using ProBond Purification System (Life Technologies, Paisley, Scotland, UK) according to manufacturer's instructions. Briefly, the cell pellet was resuspended in a guanidinium lysis buffer [6 M Guanidine Hydrochloride, 20 mM Sodium Phosphate, 500 mM NaCl, pH 7.8], flushed through an 18G syringe and transferred to a Ni-NTA agarose equilibrated with denaturing binding buffer [8 M Urea, 20 mM Sodium Phosphate, pH 7.8]. The suspension was centrifuged at 1000 rpm for 2 min, rotated for 30 min at room temperature, washed once with denaturing binding buffer and four times with denaturing wash buffer of decreasing pH [8 M Urea, 20 mM Sodium Phosphate, 500 mM NaCl, pH 6.0 and pH 5.3]. The μNS recombinant protein was eluted with an elution buffer containing 8 M Urea, 20 mM $Na_2H_2PO_4$ (pH 4.0), and 500 mM NaCl. The purity of the recombinant protein was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), using 4–12% Bis–Tris Criterion XT gel (Bio-Rad). Protein concentration was determined using the DC Protein Assay Reagent Package (Bio-Rad) at an absorbance of 650 nm, with bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, Mo) and gamma globulin as the protein standards.

The μNS control was made from cell pellet of normal sf9 insect cells (BD Bioscience). The pellet was thawed on ice. 20 μL of cells were added to 1 ml of I-PER reagent (Thermo Scientific, Rockford, USA) containing one tablet of complete ultra tablet mini (Roche, Basel, Switzerland) per 10 ml reagent. The suspended cells were pipetted several times, then vortexed for 5 s at medium speed. After 10 min incubation on ice, the sample was centrifuged at 15.000×*g* for 15 min at 4 °C. Buffer was changed using Slid-A-lyzer Dialysis Casette 3500 MWCO (Thermo scientific) in PBS overnight at 4 °C. The PBS was changed once after 5 h. The protein concentration was measured as described above for the *E.coli* background control.

2.6. Bioplex assay

MagPlex®–C Microspheres (Luminex Corp. Austin, TX, USA) #12, #21, #34 and #54 were coated using the Bio-Plex Amine Coupling Kit (Bio-Rad). Briefly, the uncoupled beads were vortexed and sonicated before $1{,}25 \times 10^6$ beads (100 μl) were transferred into tubes, washed once in wash buffer in a magnetic separator and re-suspended in bead activation buffer. Added 10 μL N-Hydroxysulfosuccinimide sodium salt and 10 μL N-(3-Dimethylaminopropyl)-N'-ethylcarbid (Both Sigma), prepared in bead activation buffer (both solutions 50 mg/mL) immediately before use, and incubated for 20 min. Beads were washed twice in PBS, before 12 μg of selected recombinant protein was added, and the volume brought up to 500 μl with activation buffer. After 2 h of incubation, beads were washed twice and resuspended in blocking buffer. After another 30 min of incubation, beads were washed in storage buffer and resuspended in 150 μl storage buffer. The bead concentrations were determined using Countess (Invitrogen). Coupled beads were stored in black Eppendorf tubes at 4 °C for up to 10 weeks. All incubations were performed in room temperature, protected from light on a rotator at 500 RPM.

For the immunoassay, Bio-Plex Pro™ Flat Bottom Plates were used. Beads were diluted in PBS containing 0,5% BSA (Rinder-albumin; Bio-Rad Diagnostics GmbH, Dreieich, Germany) and 0,05% azide (Merck, Darmstadt, Germany) (PBS+) and 5000 beads were added to each well. The beads were then washed three times. All washing steps were done with 200 μl PBS + for 30 s on a shaker on 800 rpm, then 120 s in a BioPlex handheld magnetic washer before the supernatant was poured off. Plasma samples were diluted 1:100 in PBS+ and 50 μl was added to each well. The plate was incubated on a shaker (800 rpm) for 30 min, then washed three

times in PBS+. All following incubations and washings were performed similarly. The AntiSalmonid-IgH monoclonal antibody (clone IPA5F12) (Cedarlane, Burlington, Ontario, Canada) was used as an unconjugated anti-IgM heavy chain monoclonal antibody. This mAb's reactivity against salmonid IgM heavy chain is documented by the manufacturer, and staining patterns from Hedfors et al. [35] indicates that this mAb recognizes both IgM-A and IgM-B isotypes in Atlantic salmon. AntiSalmonid-IgH was diluted in PBS + to 0,25 μg/ml and 50 μl was added to each well before incubation and washing. Biotinylated goat AntiMouse IgG2a antibody (Southern Biotechnology Association, Birmingham, AL, USA) was diluted in PBS + to 0,5 μg/ml, and 50 μl was added to each well before incubation and washing, and finally Streptavidin-PE (Invitrogen) was diluted in PBS + to 20 μg/ml, and 50 μl were added to each well before incubation and washing. 100 μl sheet fluid (Bio-Rad) was added to each well and the plate was placed on a shaker for 5 min at 800 rpm. During the whole procedure, beads were protected from light. Plates were read using a Bio-Plex 200 (Bio-Rad). The reading was carried out using a low PMT target value, the DD-gate was set to 5000–25000, and between 20 and 100 beads from each population were read from each well. Results were analysed using the BioPlex Manager 5.0 (Bio-Rad).

For verification of protein bead coupling, beads were incubated with rabbit antibodies against the recombinant proteins (Anti-μ1c K265, Anti-σ1 K275, Anti-σ3 K267 and anti-μNS [22,27].

2.7. SDS-PAGE and western blotting

Western blotting was used to verify the salmon antibodies recognizing μ1c. 5 μl of the μ1c protein sample was added to 30 μl dH_2O, 2,5 μl Reducing Agent (Bio-Rad, Hercules, CA, USA) and 12,5 μl Sample Buffer (Bio-Rad), and heated to 95 °C for 5 min before separation by gel electrophoresis (SDS-PAGE) in a 4–12% Bis-Tris Criterion™ XT PreCast Gel (Bio-Rad). Magic Mark™ XP Standard (Invitrogen) was used as protein standard. After the gel electrophoresis the protein was transferred to an Immuno-blot PVDF membrane (Bio-Rad). The membrane was blocked in PBS-SIFF with 0.001% Tween 20 (EMD Millipore) and 3% BSA for 1 h before incubation with plasma from salmon diluted 1:100 in PBS-SIFF with 0.001% Tween 20 and 1% BSA (PBS ++) overnight at 4 °C on a roller. The membrane was washed 4 × 15 min in PBS ++, and then incubated with AntiSalmonid IgH antibody (clone IPA5F12) diluted 1:500 in PBS++ for 1 h in room temperature. The washing was repeated and the membrane was incubated with Anti-Mouse IgG-HRP (GE Healthcare, Buckinghamshire, UK) diluted 1:50000 in PBS ++) for 1 h at room temperature. The signal was developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare) and detected on Bio-Rad Chemidoc XRS.

To verify that the protein amount in the background was similar or higher than the amount of *E.coli*-protein residues in the u1c sample, an SDS-page gel was run and stained with 30 mL GelCode™ Blue Safe Protein Stain (Thermo Scientific) for 20 min and washed 3 × 15 min in DEPC water.

2.8. Statistics

A Mann-Whitney test was performed to compare differences in heart pathology between challenge study time points with Stata 14.2 (Copyright 1985–2015 StataCorp LP StataCorp Texas, USA). Differences in MFI values and relative PRV levels ($2^{-\Delta\Delta Ct}$) between infected and control groups were calculated using one-way ANOVA with Tukey's multiple comparisons test. Correlation between IgM levels and relative virus RNA levels were performed using non-parametric Spearman correlation. P-values less than 0,05 were considered significant.

3. Results

3.1. Establishing the immunoassay

Recombinant PRV proteins σ1, σ3, μ1c and μNS were coupled to beads and tested against plasma collected from 8 PRV infected Atlantic salmon, 4 sampled at 12 wpc and 4 at 14 wpc (Fig. 1a) and from 4 uninfected control fish, all from a previously published PRV challenge trial [21].The μ1c coated beads bound plasma IgM strongly in 7 out of 8 infected samples and μNS coated beads in all samples, as demonstrated by mean fluorescence intensity levels (MFI) above 500 (Fig. 1). For control fish, no beads obtained values above 100 MFI after incubation in plasma (data not shown). No IgM directed against σ1 and σ3 were detected in the assay, and these were not subjected to further analysis.

A bead based immunoassay using beads coupled with recombinant PRV protein was exposed to plasma (1:100 dilution) from PRV infected Atlantic salmon (n = 8) and analysed for salmonid IgM binding using fluorescent markers. The mean fluorescent intensity (MFI) detected on beads coupled with the PRV-proteins, σ1, σ3, μ1c and μNS after plasma exposure was assessed.

Specific binding of plasma IgM to μ1c was demonstrated by western blotting using antibodies in plasma from PRV-infected fish. A protein band of approximately 75 kD was detected, close to the predicted size for μ1c (74,2 kD) [27] (Fig. 2a). No such antibodies were detected in plasma from naïve fish. Western blotting failed to demonstrate IgM-binding to μNS.

Four different concentrations of recombinant μ1c and *E. coli* background protein were tested for obtaining the best differentiation between positive and negative plasma samples, and 12 μg was chosen and used for all proteins (results not shown). In order to decide the optimal plasma dilution, eight plasma samples were analysed in different concentrations on μ1c-coated beads (Fig. 2b) and control beads (Fig. 2c). Linear dilution curves were found through 1:100 to 1:800 dilutions for all eight plasma samples. 1:100 dilution was chosen for further analysis of plasma samples due to high responses and acceptable background levels.

The recombinant μ1c protein (75 kDa) was detected by Western blot (A) using pooled plasma from eight PRV-infected fish in place of a primary antibody (+). A representative western blot is shown. The protein was not detected when plasma from control fish was used (−). Titration effects shown for plasma from seven PRV-infected fish diluted 1:100, 1:200, 1:400 and 1:800 prior to exposure to beads coated with recombinant μ1c (B) or background proteins (C).

All beads were analysed as both single- and multiplex, and gave comparable results. All further results shown are from multiplex analyses of μ1c, μNS and background protein-coated beads. A systematic variation was seen between technical replicates, suggesting a plate-to-plate variation as seen in ELISAs (Fig. S2).

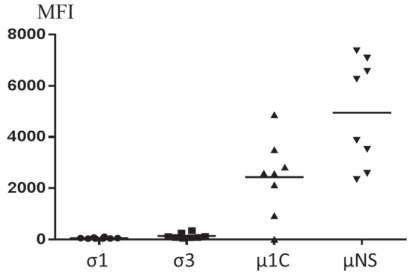

Fig. 1. Detection of PRV protein-specific IgM by bead based immunoassay.

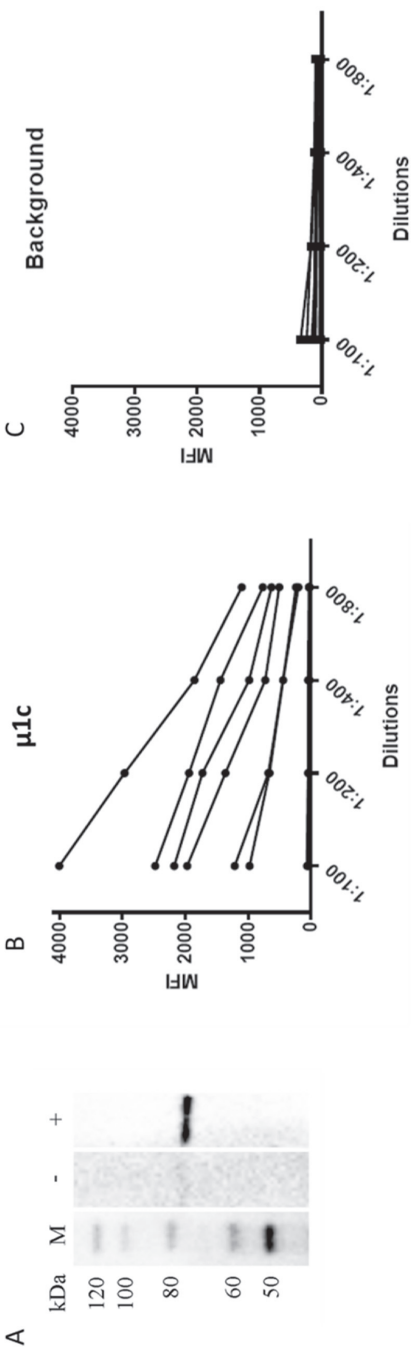

Fig. 2. Specificity and sensitivity of PRV μ1c specific IgM detection.

3.2. Verification of infection

In the cohabitation challenge study assayed, PRV infection was confirmed by RTqPCR in all individual cohabitants (n = 16 per time point) sampled from the challenged groups from 4 to 10 WPC (Fig. 3a and b). The PRV RNA values reached peak levels in blood and spleen at 4 WPC and in heart at 6 WPC. At 10 WPC, the PRV RNA levels were significantly reduced in all tissues. Histopathological evaluation of the hearts confirmed typical HSMI development in the infected fish (Fig. 3c).

PRV RNA levels assessed by RT-qPCR in blood, spleen and heart during the course of a cohabitation trial in Atlantic salmon. Crude PRV Ct levels (A) and relative PRV levels ($2^{-\Delta\Delta Ct}$) normalized for EF1 and the mean level at 4 WPC (set to 1000) (B) are shown. Red: Blood, Blue: spleen, Black: heart. In B, significant differences from 8 to 10 WPC are marked: ***P ≤ 0,001, ***P ≤ 0,0001. Histopathological scoring (C), monitored on hearts sampled week 6, 8 and 10 (n = 16 per time point) after infection of shedder fish. Green; no pathological changes, yellow; mild pathological changes, orange; moderate pathological changes, red; extensive pathological changes. Control: uninfected fish (n = 17).

All of the infected fish had detectable pathological changes in myocardium ranging from mild to extensive in contrast to the control group where only one fish had mild changes in myocardium at week eight. For epicardium, two fish in the infected groups had no classified changes in epicardium at week six and week ten. In the control group, one fish sampled at week six presented with mild epicarditis. The changes in epicardium were more extensive at 6 WPC compared to 8 and 10 weeks after infection, indicating a possible regeneration of the heart tissue. This is in line with the lower levels of virus RNA observed in the heart at 8 and 10 WPC. Pathological changes in the myocardium were observed 6, 8 and 10 WPC.

In the epicardium a reduction in histopathology score was detected at 10 WPC compared to 6 and 8 WPC. No such reduction was seen in the myocardium. No mortality occurred during the experiment.

3.3. Antibody responses in PRV infected salmon

Plasma samples from infected and control fish were analysed for antibodies against μ1c (Fig. 4a) and μNS (Fig. 4b).

Beads conjugated with PRV μ1c (A) and μNS (B) recombinant protein were incubated with plasma harvested at 0, 4, 6, 8 and 10 WPC from salmon infected with PRV by cohabitation (n = 16 per time point), and uninfected control fish (n = 8 per time point) and ten naïve fish sampled prior to infection. Mean fluorescence intensity (MFI) obtained for each individual sample on antigen coated beads were corrected for MFI on control beads, and the corrected values are displayed. Significant differences between infected and time matched control groups are marked; ***P ≤ 0,001, ***P ≤ 0,0001.

The antibody binding to background beads (background MFI) increased slightly from 0 WPC (μ1c:17–31; μNS: 40–63) to 10 WPC (μ1c: 55–310,5 and one outlier at 5667; μNS: 81–371,5). To compensate for this variation the background MFI was subtracted from the MFI obtained on μ1c and μNS- coated beads for each individual sample. This was done for all samples. All samples were analysed at least three times to confirm the accuracy of the assay. There was a significant increase in μ1-specific IgM from 8 WPC compared to uninfected control plasma, increasing further at 10 WPC. The individual variation between individuals was relatively large. For the μNS assay, the antibody binding to beads (MFI) was significantly increased compared to controls from 6 WPC, increased further at 8 WPC and then tended to plateau. A nonparametric

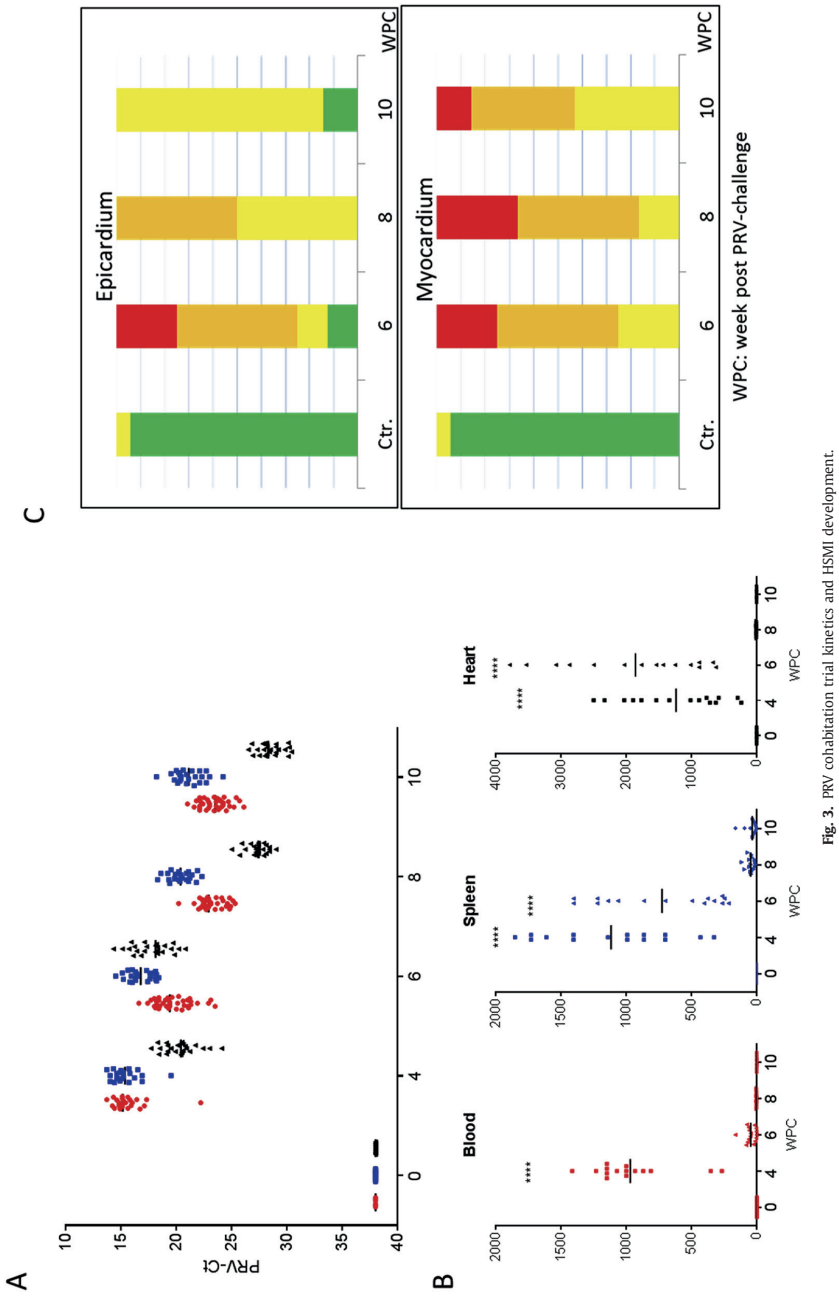

Fig. 3. PRV cohabitation trial kinetics and HSMI development.

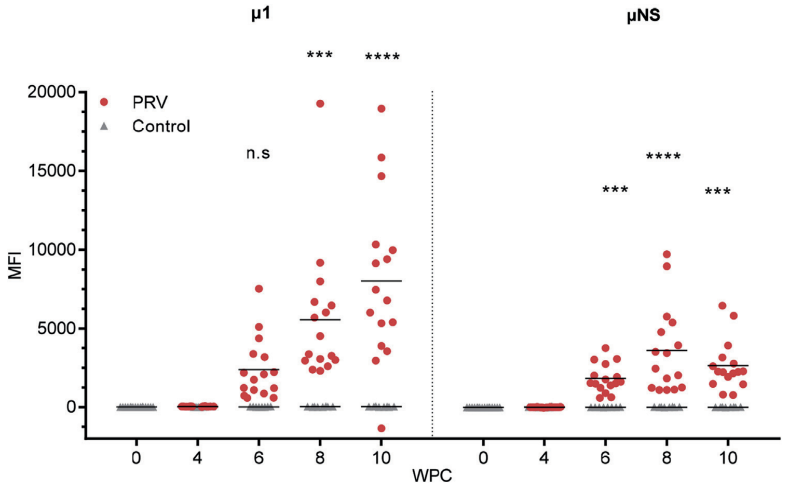

Fig. 4. Kinetics of PRV μ1c and μNS-specific IgM production.

Spearman correlation was used to compare IgM levels (based on MFI) against blood, spleen and heart PRV RNA levels in individual fish, and revealed a significant relationship between μ1 MFI and relative virus RNA levels in heart at 4 WPC ($P = 0{,}0357$, $r = 0{,}5505$), but not at other time points (Fig. S3), or in any other tissues. No correlation between μNS MFI and relative virus RNA levels was obtained (Fig. S4), or between μ1c MFI and μNS MFI at any time points (Fig. S5).

4. Discussion

Multiplexed bead based immunoassays are promising tools for specific antibody detection, and the present paper demonstrates the applicability of the assay for Atlantic salmon. Considering the time and cost saving aspects of the method, it has the potential for use in diagnostics of several diseases affecting the aquaculture industry. This report presents a bead-based assay for detection of specific antibodies against the PRV proteins μ1c and μNS in plasma of Atlantic salmon, and demonstrates a distinct increase in these specific antibodies during the course of a controlled PRV infection. The method has the potential to increase the understanding of host-pathogen interactions and humoral immune responses to infections, not only for PRV, but also for a range of other pathogens.

Multiplexed bead based immunoassays have several advantages over traditional ELISA assays. The sensitivity is higher, and antibodies against several antigens and/or pathogens can be analysed simultaneously. The increased sensitivity reduces the amount of sample needed for analysis to a few microliters. This allows 100-fold dilution of the sample, which makes artefacts and background effects due to other plasma factors less likely, and the analysis more accurate. Heat inactivation of serum/plasma is reported to further increase the sensitivity of the assay, but for high dilutions the effect of heat inactivation is minor [36].

HSMI has for more than a decade been viewed mainly as a disease problem for Norwegian salmon aquaculture. However, there is an increasing international focus on PRV due to recent findings of the virus and disease in farmed Atlantic salmon in Canada and Chile [14,15]. In addition, variants of PRV have been identified in rainbow trout and largemouth bass [37,38]. The link between PRV and HSMI has been debated, since the virus is considered ubiquitous in farmed fish, but does not always lead to disease [18,39,40]. However, several challenge studies published lately indicate that replication of PRV in salmon erythrocytes and myocytes is indeed the trigger of HSMI [21,22,41,42]. Understanding how and why HSMI develops and how the host responds to PRV has become more important than ever. A method for detecting PRV-specific antibodies in plasma and study how their production correlate with disease development is a valuable addition. Until now, no assays to identify PRV specific antibodies have been developed. The method presented here have so far only been tested in a controlled PRV challenge trial with unvaccinated fish, and it remains to elucidate if the assay is suitable for determining antibody production in naturally infected fish collected at fish farms.

Among the four recombinant PRV proteins tested for antibody binding in this study, three proteins predicted to be part of the outer PRV capsid were included; σ1, μ1c and σ3. For mammalian orthoreovirus (MRV) these proteins form the unit important for receptor-mediated endocytosis and transmembrane passage of the virus. The σ1 protein attaches to cell surface receptors and is important for the cell and tissue tropism [43,44]. Following endocytosis, the outer capsid undergoes proteolysis within the acidic compartment of the endosomes, resulting in the removal of σ3 and cleavage of μ1 [29]. The resulting intermediate subviral particles (ISVPs) penetrate the endosomal lipid bilayer, probably through the action of exposed hydrophobic parts of the cleaved μ1 protein [45]. This makes endosomal membrane penetration possible and is followed by cytoplasmic release of transcriptionally active viral cores [46]. For MRV, antibodies binding to σ1, σ3 and μ1c as well as λ2 were shown to be neutralizing [30].

Both PRV sigma proteins that were tested in our assays did not show binding of specific antibodies. It should be noted that the sequence homology between the MRV and PRV capsid proteins is very low [27], and the target cells differ [21]. For that reason, σ1 cannot be determined as the receptor binding protein for PRV. However, it is quite unlikely that the sigma proteins fail to elicit an antibody response in fish while μ1c and the non-structural μNS does so. The most likely reason we did not detect antibodies directed against these proteins is that recombinant protein produced in an *E.coli* expression system does not exhibit the native folding or post-translational modifications obtained in fish host

cells, rendering them undetectable for native antibodies. Another explanation for the negative results obtained with σ1 and σ3 recombinant proteins could be that the epitope was not available to antibody binding, either caused by the protein tag or by the bead-coupling protocol.

It is interesting to note that the μNS-protein, demonstrated to be a main scaffolding protein for the formation of cytoplasmic factories for PRV [24], is also an effective antigen for antibody formation. This protein is most likely retained in the cytoplasm and less likely to be part of the virus particle or transported out of the cell, and is therefore more likely to trigger cellular than humoral immunity. Virus-induced lysis has not been described for PRV-infected erythrocytes. Neither in cultures [25], nor as anemia in PRV infected salmon. However, previous studies and transcriptome analysis has indicated activation of cytotoxic genes in spleen and heart during the course of PRV infection [47,48], proposing that cytotoxic lysis of PRV-infected cells may occur.

The present study demonstrated a strong antibody response to PRV infection. The antibodies could be detected in plasma approximately two weeks after the first detection of virus in heart, spleen and blood. Interestingly the antibody response to the non-structural μNS protein developed prior to the response against the structural protein μ1c, and the detection (MFI) significantly differed between infected and control plasma from 6 WPC for μNS, compared to first significant difference observed at 8 WPC for μ1. This implies that the non-structural μNS protein, which is involved in viral factory formation, may be exposed to the immune system early in the infection cycle.

We have so far no information on the role of the μ1c-specific IgM, but as the antibody levels continue to increase along with the resolution of inflammation and significant reduction of histopathological changes from 6 to 10 WPC, the specific μ1c IgM response may be a contributing factor to clearance of the infection. There is a possibility that the μ1c-specific antibody may have a virus neutralizing effect like the one observed against MRV μ1. Interestingly, a negative correlation was observed between μ1c IgM levels and relative PRV RNA levels in heart at 4 wpc, but not at later time points. This could indicate that early production of μ1c antibodies limited heart infection, but in general antibody levels were very low at this time point and further studies are needed to address this question.

No other direct correlation between antibody levels, virus levels or histopathological changes could be detected in individual fish. The virus was first detected in blood and spleen, then peaked in the heart at the same time point as histopathological changes in the epicardium were detected, followed by a decrease. There are different reports on the effect of specific antibodies in different infections of salmonids. Some papers claim a good correlation between antibody titer in ELISAs and the protection acquired after a vaccine or challenge trial, including a study on infectious pancreas necrosis virus (IPNV) [3], whereas others have found no such correlation, or that the mechanism of protection is independent of the antibody level [49]. The survival from infectious salmon anemia virus (ISAV)-infection was, according to Jørgensen et al. [50], correlating more strongly to the level of cell-mediated responses than to the level of humoral responses, based on immunoglobulin gene expression. However, in that study the amount of specific antibodies was not recorded. For salmonid alphavirus (SAV) linked to pancreas disease (PD), neutralizing antibodies have been described, indicating that humoral immune responses may play an essential role in the immune response against this virus [8]. As correlation does not imply causation, reporting a correlation between antibody titres and less severe histopathology does not prove that the protection was achieved by antibodies. Other possible means by which protection was achieved could be innate antiviral immune responses or cell mediated adaptive immunity.

Characterizing and understanding the immune response against viruses is an important step in developing protective vaccines and identifying conditions, treatments or feed components which optimizes protective immune responses and host robustness to infection. The formation of specific antibodies against viral proteins shows that the humoral response is functional and activated, and the next step is to unravel the protective potential and the duration of this response. The bead-based immunoassay technology in general and the PRV antibody detection assay in particular is truly a valuable tool for studying the immune response in fish, in addition to having a diagnostic value in identifying virus exposed fish populations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.02.043.

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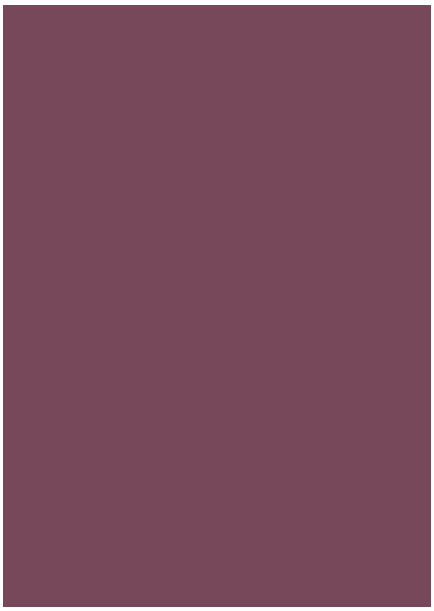
Paper II

II



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Detection of Salmonid IgM Specific to the *Piscine Orthoreovirus* Outer Capsid Spike Protein Sigma 1 Using Lipid-Modified Antigens in a Bead-Based Antibody Detection Assay

*Lena Hammerlund Teige[1], Subramani Kumar[1,2], Grethe M. Johansen[1], Øystein Wessel[1], Niccolò Vendramin[3], Morten Lund[4,5], Espen Rimstad[1], Preben Boysen[1] and Maria K. Dahle[4]**

[1] Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, Oslo, Norway, [2] Stem Cell and Cancer Biology Lab, Centre for Biotechnology, Anna University, Chennai, India, [3] National Institute of Aquatic Resources, Technical University of Denmark, Lyngby, Denmark, [4] Department of Fish Health, Norwegian Veterinary Institute, Oslo, Norway, [5] PatoGen, Alesund, Norway

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Irene Salinas, University of New Mexico, United States

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Kim Dawn Thompson, Moredun Research Institute, United Kingdom

***Correspondence:**
Maria K. Dahle
maria.dahle@vetinst.no

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Bead-based multiplex immunoassays are promising tools for determination of the specific humoral immune response. In this study, we developed a multiplexed bead-based immunoassay for the detection of Atlantic salmon (*Salmo salar*) antibodies against *Piscine orthoreovirus* (PRV). Three different genotypes of PRV (PRV-1, PRV-2, and PRV-3) cause disease in farmed salmonids. The PRV outer capsid spike protein σ1 is predicted to be a host receptor binding protein and a target for neutralizing and protective antibodies. While recombinant σ1 performed poorly as an antigen to detect specific antibodies, N-terminal lipid modification of recombinant PRV-1 σ1 enabled sensitive detection of specific IgM in the bead-based assay. The specificity of anti-PRV-1 σ1 antibodies was confirmed by western blotting and pre-adsorption of plasma. Binding of non-specific IgM to beads coated with control antigens also increased after PRV infection, indicating a release of polyreactive antibodies. This non-specific binding was reduced by heat treatment of plasma. The same immunoassay also detected anti-PRV-3 σ1 antibodies from infected rainbow trout. In summary, a refined bead based immunoassay created by N-terminal lipid-modification of the PRV-1 σ1 antigen allowed sensitive detection of anti-PRV-1 and anti-PRV-3 antibodies from salmonids.

Keywords: Atlantic salmon (*Salmo salar* L.), antibody, IgM, bead-based immunoassay, *Piscine orthoreovirus* (PRV), heart and skeletal muscle inflammation, heat inactivated plasma

INTRODUCTION

Atlantic salmon (*Salmo salar* L.) aquaculture has become an intensive and large-scale industry, and control of infectious diseases is an increasingly important task. Infectious diseases may be counteracted by vaccination, however, vaccine development against viral diseases in Atlantic salmon has not been straightforward, and few commercially available, efficient virus vaccines, are in use (1). An associated challenge has been to identify good correlates of protection, i.e., assays that can predict protective immunity (2). Important here are assays for detection of specific antibodies.

Bead-based multiplex immunoassays, such as the Luminex xMAP technology, have been successfully used to detect mammalian antibodies for more than a decade (3–5). This method has the potential to detect specific antibodies against several antigens simultaneously, and can be used to identify antibodies directed against a wide range of antigens in one sample using small amounts of antigens and sample material. According to producers, the cost of the xMAP assay is about half the cost of the same analysis using an Enzyme-Linked Immunosorbent Assays (ELISA) (www.bio-rad.com/webroot/web/pdf/lsr/literature/6313.pdf). The possibility to measure multiple analytes in the same sample further decrease the cost of each analysis. In addition to this, the xMAP assay is time-saving, can be used with much smaller sample volumes, uses around 1/50 the amount of capture antigen and offers broader dynamic range and higher sensitivity (3, 6, 7). The first bead-based multiplex immunoassays made to detect virus-specific antibodies in farmed Atlantic salmon were created and published in 2017 (8).

In mammals, the dominating circulating antibody isotype is IgG, while IgM is generally of lower affinity and comparatively more polyreactive (9); hence most assays to detect mammalian specific antibody responses target IgG. In contrast, the dominating isotype in teleost fish serum is IgM (10), requiring antibody responses to be measured within this compartment. The limited specificity of IgM is expected to give rise to detection of unspecific targets in fish, experienced as false positives in an antibody assay. Serology, i.e., detecting previous exposure to specific pathogen antigen by antibody repertoires, has not been widely used in aquaculture, but is commonly used for humans and in terrestrial animal husbandry for diagnosis and surveillance purposes. ELISAs with whole viral particles or recombinant viral proteins as capture antigen and neutralization bioassays have been used for diagnostics in aquaculture (11–15), but these methods require relatively large volumes of sample material and are time-consuming and costly when analyzing for antibodies against multiple target antigens.

Piscine orthoreovirus (PRV) belongs to the genus *Orthoreovirus* in the family *Reoviridae,* which have a segmented double-stranded RNA genome enclosed in a double-layered icosahedral capsid. Different PRV genotypes cause diseases in farmed salmonids; including PRV-1 mediated heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (16, 17), PRV-2 mediated erythrocytic inclusion body syndrome (EIBS) in coho salmon (*Onchorhynchus kisutchi*) in Japan (18), and PRV-3 mediated anemia and HSMI-like heart pathology in rainbow trout (*Onchorhynchus mykiss*) in Europe (19–22).

HSMI is one of the most prevalent diseases in farmed Atlantic salmon in Norway (16, 23, 24), and is reported from farmed salmon in several other countries as well (25–27). During the course of HSMI in Atlantic salmon, the virus peak occurs after replication in the red blood cells (24). This is followed by infection of myocytes (28), which is associated with inflammation in the heart- and skeletal red muscles (16, 17, 29). Typical histopathological signs include epi-, endo- and myocarditis, myositis, and necrosis of myocardium and red skeletal muscle (30). Mortality from HSMI varies from 0 to 20% in a net-pen, but near 100% of the fish show histopathological changes (31). Experiments have associated HSMI with reduced tolerance to hypoxic stress, which may increase mortality (32). PRV-1 is ubiquitous in farmed Atlantic salmon a few months after sea entry, presumably due to a combination of virus, host and management factors such as infectivity, host susceptibility, amounts of shedding, farms size, density of farms, and persistence of infection (33). Persistence of PRV-1 has also been associated with melanized foci in white skeletal muscle (34).

PRV-3 can infect both rainbow trout and Atlantic salmon, but with a slower replication rate and less heart pathology in salmon (20). The virus has been detected in farmed salmonids in several European countries and Chile (22, 25, 35, 36), and in wild seatrout (*Salmo trutta*) and Atlantic salmon in Norway (37). PRV-3 has an 80–90% nucleotide and amino acid sequence identity to PRV-1, and rabbit antisera raised against PRV-1 proteins cross-reacts with PRV-3 proteins (35). Secondary structure predictions also support a high conservation of protein structure between homologous PRV-1 and PRV-3 proteins (35).

The information on protein structure and function in PRV is limited. *Mammalian orthoreovirus* (MRV) has been extensively studied, and based on strong conservation of secondary structure, is used as a model for predicting PRV structure and infection cycle. Based on sequence homology to MRV and other reoviruses, a PRV particle is predicted to consist of nine proteins forming the inner and outer capsids, and there are three additional non-structural proteins involved in the replication process in the infected cell (38). In MRV, trimers of the σ1 protein form spikes in the outer capsid and is the cell attachment protein and serotype determinant (39–41). Genetic analysis of PRV indicate that σ1 is the cell attachment protein for PRV as well (38). Monoclonal antibodies directed against MRV σ1 have been shown to be neutralizing (42).

Bead-based multiplex immunoassays using recombinant outer capsid μ1c and virus-factory μNS proteins were recently used to demonstrate PRV-specific IgM in plasma from experimentally PRV-1-infected Atlantic salmon (8) and PRV-3-infected rainbow trout (21). Recombinant PRV σ1 was also tested (8), but failed to bind antibodies from plasma efficiently. The PRV σ1 spike protein is particularly interesting, as it is likely to be the receptor binding protein, and antibodies directed against epitopes on σ1 could be virus neutralizing and protective.

Common bacterial expression systems can synthetize misfolded proteins or proteins without the correct post-translational modifications. This is a likely explanation of why the previously tested PRV σ1 failed at binding antibodies in the immunoassay. Lipid modification is a natural part of post-translational modifications of proteins targeting the outer or inner membrane in gram negative bacteria (43). The lipid-modification and membrane localization can contribute to a more correct conformation of the recombinant protein compared to cytosolic production. Bacterial lipid modification is controlled via an N-terminal signal peptide in the prolipoprotein. Through the secretory and twin-arginine translocation (Sec and Tat) pathways (44), three consecutive enzymatic steps lead to modification of a cysteine residue in the signal peptide, turning

it into N-acyl S-diacylglyceryl cysteine (45). In addition to affecting the protein conformation, lipid modification can also help proteins attach to hydrophobic surfaces, like the polystyrene plastic in ELISA plates, in the right conformation via their hydrophobic lipid part. This is a potential way of improving a diagnostic immunoassay (46, 47). In this manner, an ELISA using the ICP11 protein of shrimp white spot syndrome virus (WSSV) was recently optimized using bacterial lipid modification (46).

We targeted recombinant PRV σ1 for the bacterial lipid modification system by fusing it to an N-terminal peptide containing the Tat prolipoprotein signaling sequence in the pG-TL vector, thereby targeting it for modification with an N-acyl-S-diacylglyceryl moiety (48). By coupling this modified antigen (LM-PRVσ1) to beads in the multiplex immunoassay, we were able to detect specific antibodies against PRV σ1. Here, we demonstrate the Atlantic salmon antibody response against PRV-1 σ1, and the cross-reactivity with rainbow trout antibodies against PRV-3 σ1.

MATERIALS AND METHODS

Experimental PRV-1 Infection Trial and Blood Sampling in Atlantic Salmon

Plasma for antibody detection was collected from infected and uninfected groups of Atlantic salmon (SalmoBreed strain) from a PRV-1 challenge trial described in detail in Lund et al. (32). The trial was 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).

In brief, seawater-adapted Atlantic salmon from the SalmoBreed strain (Bergen, Norway), confirmed negative for PRV and other pathogenic viruses, were kept in filtered and UV-irradiated brackish water (25‰ salinity), 12°C (±1°C) with continuous light. At Day 0, shedder fish ($N = 235$) were anesthetized (benzocaine chloride, 50 mg/L, Apotekproduksjon AS, Oslo, Norway), i.p. injected with 0.1 ml of an inoculum made from pelleted blood cells collected from a previous PRV trial (49). The virus in this material (PRV NOR2012-V3621) originates from a Norwegian field outbreak in 2012, and have been purified, characterized and used to prove causality between PRV and HSMI (17). A high level of PRV RNA was previously indicated in this material (PRV RTqPCR Ct 17.3 using a 100 ng RNA input), and the material was previously aliquoted in several batches and frozen for use in future trials (32, 49). Injected fish were placed in an experimental fish tank (1,000 L), and an equal number of naïve cohabitants was added. An identical control tank contained the same total number of uninfected fish. The infection trial lasted for 15 weeks. Ten cohabitant fish and ten control fish were sampled at 0, 4, 7, 10, 12, and 15 weeks, respectively, during which PRV infection was verified by RTqPCR, and HSMI by histological examination (32).

For sampling, the fish were euthanized by bath immersion with benzocaine chloride (200 mg/L water) (Apotekproduksjon AS, Oslo, Norway). Blood was collected from the caudal vein using lithium heparin-coated vacutainers (BD Vacutainer) with 20 G Venoject needles and centrifuged (3,000 rpm, 10 min, 4°C) for collection of plasma. The plasma samples were stored at −20°C.

Field Samples From Rainbow Trout

In January 2018, a recirculating aquaculture system farm in Jutland, Denmark, rearing rainbow trout experienced clinical disease associated with PRV-3. The Danish isolate of PRV-3 described in Dhamotharan et al. (35) was detected in heart and spleen samples from clinically affected fish by qPCR described in Finstad et al. (24), Blood samples were collected from the caudal vein of survivor fish ($N = 16$) in a raceway where clinical disease had occurred 2 months earlier.

Experimental PRV-3 Infection Trial and Blood Sampling in Rainbow Trout

The blood/plasma samples from rainbow trout was from a previously published challenge trial (20). In short, Specific Pathogen free (SPF) rainbow trout of 32 g in average were either i.p. injected with 0.1 ml of challenge inoculum or challenged by 1:1 cohabitation with the injected fish (cohabitants). The challenge inoculum was pooled rainbow trout blood (diluted 1:4 v/v in L-15 medium) from a pilot challenge study, which represented the first passage in experimental fish (20). The original material was collected from three individual fish from a rainbow trout hatchery outbreak in Norway in 2014 (19), and the PRV-3 isolate (NOR060214) has been purified, fully sequenced (35), and used in two previous experimental trials (20, 21). Blood samples were collected from eight fish sampled at 8 and 10 weeks after infection, and from eight uninfected control fish.

Construction of Plasmids for Recombinant Unmodified and Lipid-Modified PRV Protein Production

The unmodified recombinant PRV-1 σ1 and μ1c proteins were produced in *E. coli* from pcDNA3 as described by Finstad et al. (28). For lipid modified protein production, the complete open reading frame of PRV-1 σ1 gene target was obtained through PCR amplification from pcDNA3/PRV σ1 [NOR050607 (38)] using PfuUltra II Fusion HS DNA Polymerase (Agilent, Santa Clara, CA, USA). The gene specific forward and reverse primers used for amplification contained BamHI and EcoRI restriction sites at the N- and C-terminus, respectively. The PCR amplicon was resolved in 1% (w/v) agarose gel electrophoresis alongside 1 kbp DNA ladder (Fermentas Life Sciences, Germany) (**Figure S1A**) and purified according to instructions for the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL, Düren, Germany). The DNA eluates were quantified using a Nanodrop Spectrophotometer (Thermo Fisher, Wilmington, DE, USA) and cloned into the digested pG-T-LM vector containing the Tat signaling peptide (**Figure S1B**), as described earlier (48), using the In-Fusion HD cloning system (Clontech, Mountain View, CA, USA). All the recombinant constructs were screened by colony PCR using gene and vector specific primers, and further confirmed by DNA sequencing (ATCG, Toronto, Canada). The resulting

recombinant construct was named pGT-LM/PRVσ1. The lipid modification process is previously described in detail for the WSSV-ICP11 protein (48).

To be used as control antigen in this study, the unmodified and lipid modified ISAV-FP protein were produced in the same manner as the previously published WSSV-ICP11 protein (41), which was also used as a control antigen here. In brief, the complete open reading frame of the ISAV-FP gene was PCR amplified (777 bp) using gene specific primers with Nde1/EcoR1 and BamH1/ EcoR1 restriction sites at the N- and C-terminus, respectively. The targeted ISAV-FP PCR amplicons were digested using respective endonucleases. The amplicons were cloned into pET28a and pGT-LM vectors for targeted unmodified and lipid-modified protein expression, respectively. The unmodified and lipid-modified clones were verified by restriction digestion and sequencing. The expression vectors were named pET28a- ISAV-FP (unmodified) and pGT-LM-ISAV-FP (lipid- modified).

Expression of Proteins in *E. coli*

Both unmodified and lipid-modified recombinant constructs were transformed into the *E. coli* strain, GJ1158 (Genei, Bangalore, India) for protein expression. Transformants confirmed to contain the correct plasmid sequence were inoculated into 10 ml LB medium containing 100 μg/ml ampicillin, and incubated (200 rpm, 37°C) until absorbance reached 0.6 at 600 nm. Protein production was induced by adding 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and the bacterial culture was given a 4 h post-induction time (200 rpm, 37°C). The induced bacteria were harvested by centrifugation (3,000 × g, 5 min), washed twice with 0.9% saline and re-suspended in 1X phosphate buffered saline. Lysed recombinant bacteria (25 μl) were analyzed by gel electrophoresis and western blotting for recombinant lipid modified protein expression using anti-his antibodies (**Figure S2**).

Purification of Recombinant Lipid-Modified Proteins

The pelleted bacteria were re-suspended in 50 mM Sodium phosphate pH 8.0/300 mM NaCl and lysed with lysozyme (Thermo Fisher Scientific) at a final concentration 100 μg/mL for 1 h at 4°C, followed by sonication. The membrane fraction was harvested by centrifugation at 150,000 × g for 1 h at 4°C. The membrane pellet was re-suspended in lysis buffer and solubilized with 1% Sodium lauroyl sarcosinate (also known as sarkosyl) buffer (Sigma Aldrich, St. Louis, MO, USA), followed by centrifugation (1 h, 100,000 × g, 4°C). The proteins contained a 6x Histidine tag, which was utilized for purification using immobilized metal affinity chromatography (IMAC). The supernatants containing solubilized membrane proteins were loaded on a Tris-carboxymethyl ethylene diamine (TED) column pre-charged with Ni2+ ion and pre-equilibrated with equilibration buffer (MACHEREY-NAGEL). The column was then washed with wash buffer containing 5 mM imidazole. The column bound-proteins were eluted with purification buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) supplemented with 25–50 mM imidazole. The protein eluates were analyzed using Criterion precast gels (4–12%) (Bio-Rad) (**Figure 1A**).

Bead-Based Assay

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 both Sigma-Aldrich. For each coupling reaction, 6-24 μg of recombinant protein was used. Proteins used were PRV σ1, lipid modified PRV σ1 (LM-PRVσ1), lipid modified WSSV ICP11 (LM-WSSV-ICP11) unmodified infectious salmon anemia virus fusion protein (ISAV-FP), lipid modified ISAV-FP (LM-ISAV-FP) and the hapten-carrier DNP-keyhole limpet hemocyanin (DNP-KLH) (Calbiochem, Merck, Darmstadt, Germany), which represents a model antigen to estimate non-specific antibodies (50). The bead concentrations were determined using Countess automated cell counter (Invitrogen, Carlsbad, CA, USA). Coupled beads were stored in black Eppendorf tubes at 4°C for up to 10 weeks. All incubations were performed at room temperature, 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 PBS containing 0.5% BSA (Rinderalbumin; Bio-Rad Diagnostics GmbH, Dreieich, Germany) and 0.05% azide (Merck, Darmstadt, Germany) (PBS+) and 2,500 beads of each bead number were added to each well. AntiSalmonid-IgH monoclonal antibody (clone IPA5F12) (Cedarlane, Burlington, Ontario, 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 1:1,000 in PBS+ was used as a secondary antibody and Streptavidin-PE (Invitrogen) diluted 1:50 in PBS+ as the reporter flourochrome. Plates were read using a Bio-Plex 200 (Bio-Rad). The DD-gate was set to 5,000–25,000, and between 20 and 100 beads from each population were read from each well. The reading was carried out using a low PMT target value. Results were analyzed using the Bio-Plex Manager 5.0 and 6.1 (Bio-Rad).

SDS-PAGE and Western Blotting

Western blotting was used to confirm antibody binding to the specific proteins. Protein samples with the recombinant unmodified PRV-1 proteins μ1c and σ1 used previously (8), LM-PRVσ1, LM-WSSV-ICP11, ISAV-FP, and LM-ISAV-FP were analyzed. Protein concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific). From the proteins above, 0.6 μg protein was diluted to 35 μl with dH_2O. 2.5 μl Reducing Agent (Bio-Rad) and 12.5 μl Sample Buffer (Bio-Rad) was added, and the mix was heated to 95°C for 5 min before separation by gel electrophoresis (SDS-PAGE) in a 4–12% Bis-Tris Criterion™ XT PreCast

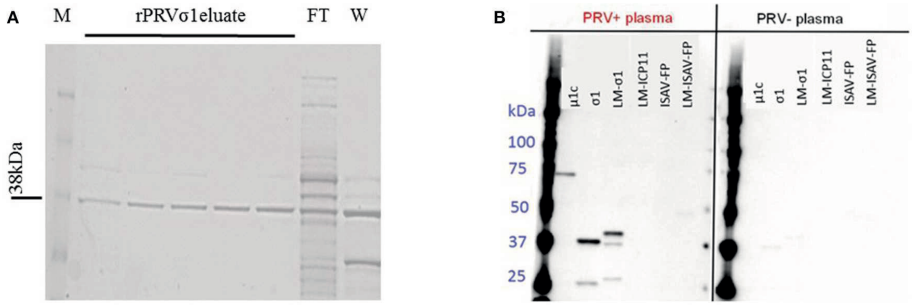

FIGURE 1 | Production of lipid modified PRV proteins. **(A)** SDS-PAGE profile showing the purified lipid-modified PRVσ1 (M-Marker, FT-Flow through, and W-Wash). Eluates 1–2 with 25 mM imidazole and 3–5 with 50 mM imidazole elution. **(B)** Specificity of plasma IgM to PRV antigens. Proteins used in the multiplex immunoassay were analyzed by western blotting. Left panel incubated with plasma from PRV-infected Atlantic salmon (pooled sample from 10 to 15 wpc) and right panel incubated with plasma from control fish (pooled samples from the same challenge trial) as primary antibody.

Gel (Bio-Rad). Precision Plus Protein Standard (Bio-Rad) was used to confirm protein size. After the gel electrophoresis, the protein was transferred to membrane using a Trans-Blot midi transfer pack (Bio-Rad). The membrane was blocked in PBS with 0.001% Tween 20 (EMD Millipore) and 5% skim milk powder (Merck) for 1 h before incubation with pooled plasma from PRV negative salmon or PRV infected salmon (0 wpc and 10–15 wpc from the PRV-1 challenge trial) diluted 1:100 overnight at 4°C on a roller. The membrane was washed 4 × 15 min, and then incubated with Anti-Salmonid IgH antibody (clone IPA5F12) (1:500) for 1 h in room temperature. The washing was repeated and the membrane was incubated with Anti-Mouse IgG-HRP ECL peroxidase-labeled Anti-Mouse antibody, NA931VS (GE Healthcare, Buckinghamshire, UK) (1:50,000) and Precision Protein StrepTactinHRP (Bio-Rad) (0.7 µl in 10 ml) for 1 h at room temperature. All antibodies were diluted in PBS with 0.001% Tween 20 and 1% skim milk powder, and all washing were done with in PBS with 0.001% Tween 20. The signal was developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare) and detected on Bio-Rad Chemidoc XRS.

Heat Treatment and Adsorption of Plasma

Aiming to eliminate background binding of plasma to non-PRV proteins, the plasma was heated to temperatures from 30 to 56°C for 5–60 min. This is in line with previously used protocols for salmon plasma complement inactivation (51, 52).

To demonstrate PRV σ1 specificity, PRV-1 positive plasma (from 12 to 15 wpc in the PRV-1 challenge trial) was adsorbed against beads coated with lipid-modified and non-lipid-modified proteins. In addition to antigens described earlier, beads coated with PRV µNS expressed in insect cells (8, 53) and *E. coli* protein (background) coated beads described earlier (8) were included in the experiment. Pooled heat-treated plasma (48°C for 20 min) was diluted 1:200, and 50 µl of each plasma sample was added to a 96 well-plate and incubated with beads. The

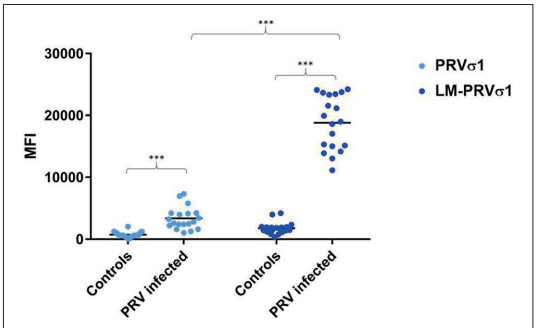

FIGURE 2 | Increased detection of anti-σ1 using lipid modified antigen. Comparison between beads coated with σ1 and beads coated with LM-σ1 when used in the immunoassay to analyze plasma from PRV-infected Atlantic salmon (12–15 wpc) and controls. Significant difference between infected and control fish, and between different antigens when used to analyse infected fish samples are indicated by asterisks (Mann-Whitney test).

beads used were coated with PRVσ1 PRVµ1c, PRVµNS, LM-WSSV-ICP11, LM-PRVσ1, ISAV-FP, or LM-ISAV-FP. Coated beads of each bead type (100,000 in 50 µl) or 50 µl PBS without beads were added per well. Incubation was done on a shaker at 500 rpm in room temperature and protected from light for 3 h. After incubation, the beads were removed using a magnetic separator, and bead-free plasma was transferred to a new plate and stored overnight at 4°C. The plasma was analyzed the next day using Bio-Plex 200 and Bioplex manager 6.1 with DNP-KLH, LM-WSSV-ICP11, LM-PRVσ1, ISAV-FP, and LM-ISAV-FP coated beads.

Statistical Analysis

A non-parametric Mann-Whitney unpaired rank test was performed between groups in **Figure 2**, between control groups and infected groups at all time points in **Figure 3** and between LM-PRVσ1 and the other proteins in **Figure 5A**. All statistical

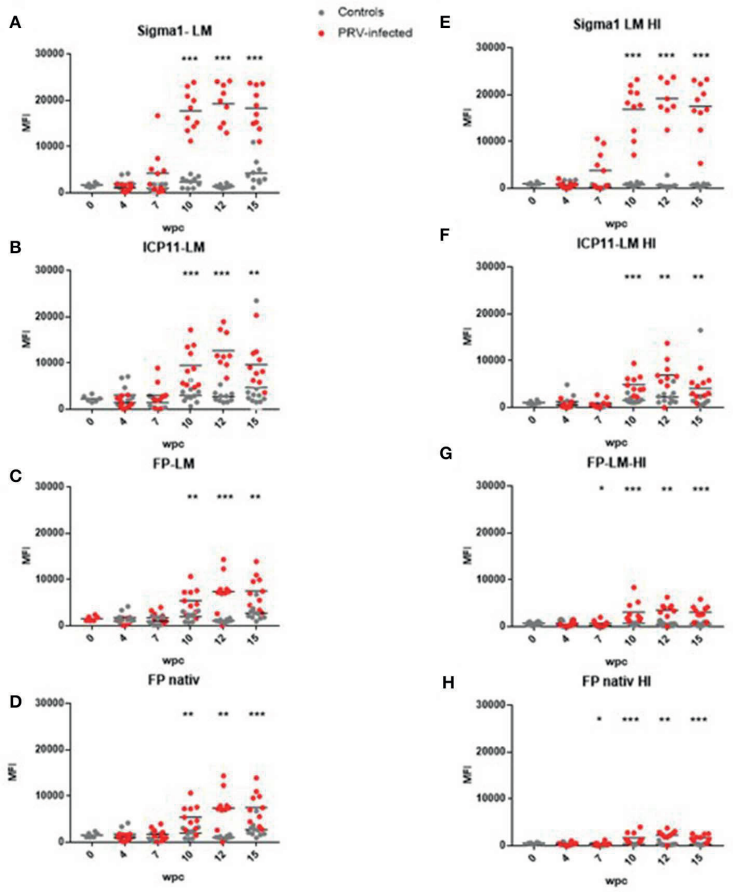

FIGURE 3 | Antibody detection in untreated and heat-treated plasma. **(A)** LM-PRVσ1 on untreated plasma. **(B)** LM-WSSV-ICP11 on untreated plasma. **(C)** LM-ISAV-FP on untreated plasma. **(D)** ISAV-FP on untreated plasma. **(E)** LM-PRVσ1 on heat-treated plasma. **(F)** LM-WSSV-ICP11 on heat-treated plasma. **(G)** LM-ISAV-FP on heat-treated plasma. **(H)** ISAV-FP on heat-treated plasma. Significant difference between groups are indicated by asterisks (Mann-Whitney test).

analyses were performed with the help of GraphPad Prism 7.03 (GraphPad Software Inc., USA).

RESULTS

Production and Purification of Lipid Modified PRV σ1

The lipid modified LM-PRVσ1 was cloned and produced in *E. coli*, and found to be located in the outer membrane of the bacteria, as confirmed through sub-cellular fractionation and western-immunoblotting (**Figure S2**). The LM-PRVσ1 was purified in a detergent-free form in a single step using immobilized metal affinity chromatography (IMAC), as previously described (48). The protein was successfully purified and a band was detected at the expected size of 38 kDa (**Figure 1A**, **Figure S3A**).

Confirmation of Anti PRV Antibody Specificity Through Immunoblotting

To show the formation of anti-PRV σ1 antibodies in PRV-infected fish, recombinant PRV σ1 protein with or without lipid modification along with PRV-1 μ1c were immunoblotted using plasma from PRV-1 infected and uninfected Atlantic salmon as a source of primary antibody. IgM binding to proteins corresponding in size to PRV σ1 and LM-PRVσ1, as well as PRVμ1c was confirmed in plasma from PRV infected fish. No

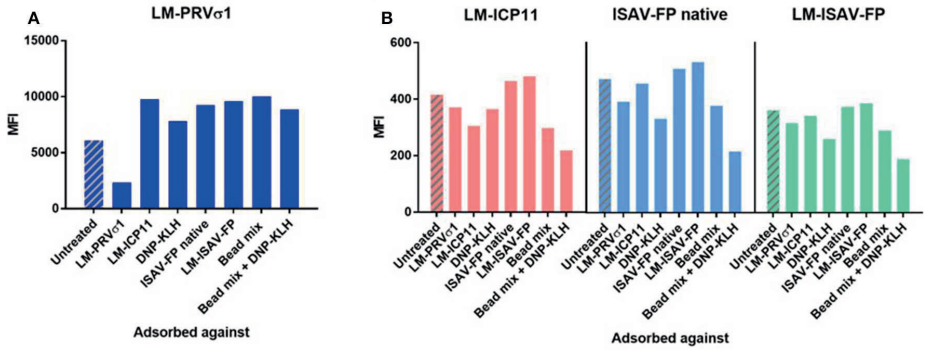

FIGURE 4 | Pre-adsorption of heat-treated pooled plasma from PRV-infected fish against antigens indicate specificity of anti-PRVσ1 antibodies. Measured on beads coupled with **(A)** LM-PRVσ1 and **(B)** non-PRV control proteins.

binding to the control antigens LM-WSSV-ICP11, LM-ISAV-FP, or ISAV-FP were observed (**Figure 1B**, **Figure S3B**). This confirms the presence of antibodies binding to σ1 in plasma from PRV-infected fish.

Lipid-Modified PRV σ1 Coated on Luminex xMAP Beads Can Be Used to Detect Anti-PRV Antibodies

Compared to unmodified PRV-1 σ1, the lipid modified PRV σ1 protein coated on xMAP beads bound the antibodies produced after PRV infection more effectively, as indicated by significantly higher levels of mean fluorescence intensity (MFI) in the luminex assay (**Figure 2**).

Anti-PRV-1 σ1 antibodies were then measured in plasma originating from a PRV-1 infection trial. In this trial, anti-PRV σ1 antibody levels increased from week 7 after PRV infection and reached a plateau at 10–15 wpc (**Figure 3A**).

Test of Binding Specificity Using Lipid-Modified Control Proteins

Other lipid-modified and unmodified proteins were tested to confirm that the antibodies binding to LM-PRVσ1 were specific for the virus protein and not targeting the N-terminal lipid modification. The control proteins used were lipid modified ICP11 from WSSV, and unmodified and lipid modified ISAV-FP. When testing the control antigens on plasma from the PRV-1 challenge trial, we observed an increase in antibodies binding to both unmodified and lipid modified proteins from week 10 after PRV challenge (**Figures 3B–D**).

Effects of Heat Treatment and Pre-adsorption of Plasma on Binding Specificity

After heat treatment of plasma to eliminate background binding to non-PRV proteins, 48°C for 20 min was found as optimal (**Figures S4A,B**). Using these treatment conditions, antibody binding to LM-PRVσ1 beads decreased using plasma from control fish, but not when using plasma from infected fish, indicating antigen specificity after infection (**Figure 3E**). For the non-PRV proteins, antibody binding decreased after heat treatment when using plasma from both infected and uninfected fish (**Figures 3F–H**). When heat-treated and untreated plasma from controls from the same individuals, sampled 12 and 15 wpc, were run on the same plate (to avoid plate-to-plate variation), the binding to LM-PRVσ1-coated beads decreased for all control fish after heat treatment. For infected fish, the antibody binding to LM-PRVσ1 decreased in some individuals and increased in others after heat treatment (**Figure S4C**).

To further evaluate the antigen specificity of the antibodies, pooled plasma was pre-adsorbed with beads coated with the specific antigens, as well as mixes of antigen-coated beads. The binding to LM-PRVσ1-coated beads decreased only after pre-adsorption of plasma with LM-PRVσ1 beads, but increased after adsorption with any of the other beads coated with LM-modified or unmodified proteins, including the hapten-carrier conjugate DNP-KLH (**Figure 4A**). Less changes were seen when analyzing binding to LM-WSSV-ICP11, LM-ISAV-FP, or ISAV-FP after adsorption, but decreases in binding were seen especially after adsorption with DNP-KLH and bead mixes (**Figure 4B**).

Anti-PRV-3 σ1 Antibodies Bind to PRV-1 σ1 LM-Coated Beads

Heat-treated plasma samples from a field outbreak of PRV-3 were analyzed using beads coated with LM-PRVσ1 as well as PRV μ1c, PRV μNS and *E. coli* protein (background) coated beads. Results show that antibody binding (MFI) to LM-PRVσ1 was significantly higher than binding to PRV μNS coated beads, PRV μ1c-coated beads as well as *E. coli* protein (background) coated beads (**Figure 5A**). LM-PRVσ1 and LM-WSSV-ICP11 beads were tested on heat-treated plasma and blood from naïve and PRV-3 infected rainbow trout. The IgM binding to LM-PRVσ1-coated beads was low in naïve fish, whereas MFI levels above 20,000 was obtained from week 10 after infection (**Figure 5B**)

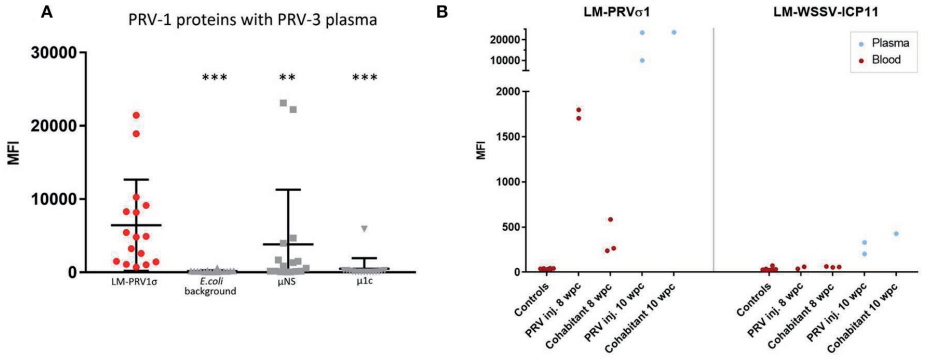

FIGURE 5 | Detection of cross-binding antibodies induced by PRV3 infection in rainbow trout. **(A)** Field samples from a PRV-3 outbreak in rainbow trout analyzed with LM-PRVσ1, μNS, and μ1c (PRV-1) coupled beads in the immunoassay. Significant difference between MFI of LM-PRVσ1 beads and the other beads are indicated by asterisks (Mann-Whitney test). **(B)** MFI from blood and plasma samples from individuals experimentally infected with PRV and uninfected controls using LM-PRVσ1 and LM-WSSV-ICP11 coupled beads. All samples were heat-treated.

Only low levels (MFI up to 426) of antibodies binding to LM-WSSV-ICP11 beads were detected (**Figure 5B**). An alignment between the σ1 amino acid sequences of PRV-1 NOR050607 coated on the beads and PRV-3 NOR060214 used in the PRV-3 infection trial revealed 81% identity (**Figure S5A**). The N-terminal was the least variable part of the protein, whereas several areas of variation were found in the central and C-terminal part. The last two AA in the C-terminal are hydrophobic in PRV-1, but hydrophilic in PRV-3. The PRV-3 sequence was 1 amino acid longer due to an inserted glycine at position 39. An antigenicity plot indicated minor differences in the antigenicity pattern between the two PRV genotypes (**Figure S5B**).

DISCUSSION

Since the σ1 protein from MRV is known for its role in receptor binding and cell entry (39, 41), and is a primary target for neutralizing antibodies (40, 54), σ1 was predicted as a promising target for neutralizing antibodies against PRV. Virus neutralization assays have been successfully used for other salmonid viruses, including the salmonid alphavirus (SAV) (55). However, no such assays have been developed for PRV, as the virus has resisted cultivation in cell lines. So far, primary erythrocytes are the only cells where PRV is reported to replicate for more than one passage *ex vivo* (56), and even in erythrocytes the consistency of replication is too low to allow the establishment of a neutralization assay. Because of this, other assays for detection of anti-PRV antibodies are attractive.

In our former development of bead based multiplex immunoassays for detection of PRV-specific antibodies we were able to detect specific IgM targeting PRV-1 μ1c and μNS proteins in Atlantic salmon plasma, but not IgM directed against the PRV-1 spike protein σ1 (8). The PRV-3 genotype has been found associated with disease in several European countries after its initial discovery in Norwegian farmed rainbow trout. In a recently published challenge trial (21), antibodies against PRV-3 μ1c were detected at low levels using a bead-based assay coated with PRV-1 μ1c. This study demonstrates that sensitive detection of anti-PRV σ1 antibodies in Atlantic salmon and anti-PRV-3 σ1 antibodies in rainbow trout was obtained through N-terminal lipid modification of the recombinant PRV σ1 antigen (LM-PRVσ1) prior to use in the bead-based immunoassay.

Lipid modification using a bacterial prolipoprotein signaling sequence have previously been put forward as a desired strategy for inducing a potential adjuvant effect to a vaccine antigen (48). In this case, we tested if the lipid-modification of recombinant PRV σ1 coated on beads could promote detection of PRV σ1-specific antibodies, and found that the lipid modification indeed led to increased antibody detection. A similar improvement of antigen-antibody interaction has been associated with increased hydrophobic anchorage of N-terminal lipid-modified antigens in other studies (47, 48). A possible reason for the improved IgM detection obtained by PRV σ1 lipid-modification is a stabilization of σ1 mimicking the conformation and/or orientation in the intact virus with the N-terminal bound to the surface and the C-terminal exposed (57). This orientation is likely to improve the exposure of the correct epitopes for detection by antibodies, including neutralizing antibodies.

For control of antigen specificity, the lipid modified ICP11 protein from the shrimp virus WSSV (58), and the fusion protein (FP) of ISAV (59), with and without lipid modification, was tested. The experimental fish had not been previously exposed to these viral proteins, as the trial fish were tested negative for ISAV (32), and WSSV is a crustacean virus (60). Nevertheless, we detected IgM binding to these proteins in salmonid plasma in uninfected fish, and this binding increased significantly during the

course of PRV infection. We also detected binding to LM-PRVσ1 in control fish not previously exposed to PRV. This background binding could be explained by polyreactive antibodies present in control fish, with increasing levels induced by the PRV infection. An induction of polyreactive antibodies after infection has been described in fish (50, 61, 62) and mammals (9).

Heat treatment of plasma at more than 43°C for as little as 5 min removed most of the background binding in control fish without reducing the specific interaction with lipid-modified PRV σ1 in infected fish, clearly indicating that PRV σ1-specific antibodies were detected. Binding to the non-PRV proteins was reduced by heat treatment, but not completely removed, and was still significantly higher in infected fish than in control fish. In contrast to the rigid structure of the classic antibody model, it has been hypothesized that polyreactive antibodies have more flexible antigen binding sites and are able to change conformation to accommodate different antigens (9). It is conceivable that heat treatment might negatively affect this flexibility or that the polyreactive antibodies is more heat-labile than the specific antibodies for other unknown reasons. Whether background binding was caused by polyreactive antibodies alone or secondary via other plasma factors, requires further study. As the lipid-modified signaling peptide fused to the PRV σ1 N-terminal is a natural part of gram negative bacterial membrane proteins (43), previous exposure to and acquired immunity against it cannot be completely ruled out. However, results from adsorption against other lipid-modified proteins indicate that antibodies detected on the LM-PRVσ1-coated beads do not bind to the acylated N-terminal peptide, but specifically to PRV σ1. Together the effects of heat treatment and pre-adsorption of plasma strongly suggest an increase in the formation of polyreactive antibodies during a PRV infection, whereas antibodies binding to the LM-PRVσ1 coated beads are PRV σ1 specific.

In the PRV-1 trial in Atlantic salmon analyzed here, PRV RNA peaked in cohabitant fish at 7 weeks post-introduction of virus shedders and histopathological changes consistent with HSMI were most prominent after 10 weeks (32). Anti-PRV σ1 IgM was produced 7 weeks after the initial exposure of experimental fish to PRV shedders, which corresponds to 3 weeks after the first detection of PRV in blood from these fish (32). This timing resembles our previous observations on production of IgM targeting the PRV μ1 and μNS proteins (8). In both the trial analyzed here, and the trial analyzed with bead based immunoassay previously (8), a reduction in HSMI lesions was observed in the time after the specific IgM production reached a maximum level, and could indicate a protective effect. Antibody-mediated protection against viruses represent the humoral arm of the adaptive immune system, but cellular protection mediated by T-lymphocytes may be equally important. Results from earlier PRV infection trials have indicated a role of cytotoxic (CD8+) T-cell mediated protection (29, 63). In particular, recruitment of immune cells to the PRV-infected heart has been associated with a reduction in PRV-infected cardiomyocytes (24, 28). This suggests a possible role for both humoral and cellular immune mechanisms in clearing of the PRV infection in the heart, and we should be careful with drawing conclusions based on correlation between specific antibody production and protection from HSMI. PRV is a virus that persists in blood cells after infection (33, 64). Viral RNA persisted in blood throughout this trial as well, showing the insufficiency of the humoral immune response to eradicate virus from blood. The IgM level stayed elevated through the duration of this study (15 weeks). Since PRV-1 causes a persistent infection in Atlantic salmon, the virus-specific IgM response can be expected to be of longer duration than shown here. Longer trials should be performed to clarify the long-term antibody production level.

We have demonstrated that LM-PRVσ1 provide a more sensitive assay for PRV-3 antibody detection than μ1c, and is more suitable for identifying populations previously exposed to PRV-3 and effects of potential vaccines. The LM-PRVσ1 assay worked in both PRV-1 infected Atlantic salmon and PRV-3 infected rainbow trout and the PRVμ1c assay worked in PRV-1 infected Atlantic salmon only (except in one fish). Multiplexing these assays can potentially be used to distinguish between infections with PRV-1 and PRV-3 in a population. PRV-1 and PRV-3 have 80.1% nucleotide and a 90.5% amino acid identity [(35); **Figure S5A**]. The similarity is somewhat higher in the N-terminal compared to the protein body and C-terminal head. Several of the amino acid differences represent significant alterations in the side chain charges or polarity, which may affect 3D structure or protein-protein interaction. The two very last C-terminal amino acids differs, containing hydrophobic side chains (isoleucines, I) in PRV-1 and polar/charged side chains [Threonine (T), arginine (R)] in PRV-3, which is likely to lead to structure and antibody epitope differences. The amino acid differences within the core of PRV σ1 differ, but clearly not enough to hamper the antibody cross-binding capacity. The functional importance of these differences are difficult to predict, as the amino acid identity between the PRV-1/-3 σ1 sequences and the MRV σ1 sequence are only approximately 21% (38). MRV σ1 is considerably larger (459 AA compared to 314 AA for PRV-1 σ1), and the extended sequence of MRV is located both in the N-and C-terminal. Based on structural analyses on MRV σ1 (54, 57, 65), it is the N-terminal tail which inserts into the virion, the body which contains the motif for sialic acids/glucans, and the C-terminal head domain which binds the target cell receptor, junctional adhesion-molecule-A (JAM-A). Neutralizing antibody binding has been localized to the C-terminal head domain (54). This part of σ1 is truncated in all PRV genotypes compared to MRV, and functional and interaction prediction *in silico* is not straightforward. The only conserved motif predicted in PRV (both genotype 1 and 3) is the glucan/sialic acid biding motif (38, 66).

In contrast to PRV-1, which establish a persistent infection that can be detected in the host up to a year after infection (64), PRV-3 is cleared from infected rainbow trout (20, 21), and an immunoassay to identify immunized populations could be particularly useful. A still open question is the duration of the specific humoral response to infection, and the possibility to identify vaccinated or previously exposed populations after more than 15 weeks.

Recently, two PRV vaccine trials using whole virus vaccines and DNA vaccines, respectively, showed partial protection of Atlantic salmon from HSMI (67, 68). In order to optimize such trials, assays that can reveal true correlates of protective immune responses against PRV are useful. Sensitive immunoassays that require small volumes of minimal-invasive samples are attractive for aquaculture. Using this bead-based detection assay, 1 µl plasma in 100-fold dilution is sufficient for providing sensitive antibody detection, and through multiplexing, a larger repertoire of pathogen-specific antibodies can be analyzed simultaneously. The potential of bead–based analyses is that not only antibody detection, but also pathogen detection and detection of other molecular markers can be obtained in concert in the same sample. As also put forward by others (69), this analytic method has a great future potential in aquacultural diagnostics.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

LT: study conception and design, acquisition of data, analysis, interpretation of data, drafting, revising, and approving the manuscript. SK: study design, acquisition of data, analysis, interpretation of data, drafting, revising, and approving the manuscript. GJ: acquisition of data, analysis, interpretation of data, revising, and approving the manuscript. ØW: interpretation of data, revising, and approving the manuscript. NV and ML: sample collection, interpretation of data, revising, and approving the manuscript. ER: study conception and design, revising, and approving the manuscript. PB: study design, interpretation of data, revising, and approving the manuscript. MD: study conception and design, analysis, interpretation of data, drafting, revising, and approving the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02119/full#supplementary-material

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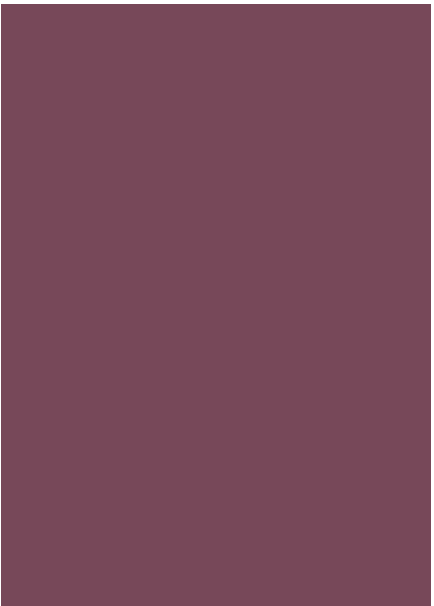
Paper III

III



IgM directed against Salmonid alphavirus antigens can be detected after a natural pancreas disease outbreak, using a bead-based immunoassay

Lena Hammerlund Teige[*1], Ida Aksnes[*1], Magnus Vikan Røsæg[2], Ingvill Jensen[3], Jorunn Jørgensen[3], Hilde Sindre[4], Espen Rimstad[1], Maria K. Dahle[3,4], Preben Boysen[1]

[1] Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, Oslo, Norway
[2] SalMar Farming AS, Trondheim, Norway
[3] The Norwegian College of Fishery Science, UiT The Arctic University of Norway, Tromsø, Norway
[4] Department of Fish Health, Norwegian Veterinary Institute, Oslo, Norway

[*]Equal contributions

Corresponding author: Preben Boysen (preben.boysen@nmbu.no)

ABSTRACT

Salmonid alphavirus (SAV) is the etiological cause of Pancreas disease (PD) in Atlantic salmon (*Salmo salar*). To monitor and control the distribution of PD in Norway, farmed salmonids in the marine phase are regularly screened for the presence of SAV by RT-qPCR. While RT-qPCR is suitable for detection of SAV in the early stages of infection, serological methods could be more suitable in the later stages or in subclinical infections. SAV neutralization assays have been traditionally used, but are time-consuming and cumbersome, thus alternative methods of antibody detection are warranted. Enzyme-linked immunosorbent assays (ELISAs) for SAV infection have not yet been successfully used for field samples. Using detergent-treated SAV particles as antigens in a bead-based immunoassay, we detected SAV-specific antibodies in plasma collected in both a SAV challenge trial and a field outbreak of PD. The background was in general low in both the challenge trial and in the outbreak. Increased levels of SAV-specific antibodies were detected, even in samples negative for viral RNA. This method is time-saving compared to neutralization assays, suitable for non-lethal testing of many individuals, and in the later stages after infection better suited than detection of virus by RT-qPCR. We conclude that the bead-based immunoassay using disrupted SAV particles is a promising diagnostic tool to complement RT-qPCR.

INTRODUCTION

Salmonid alphavirus (SAV), formally named *Salmon pancreas disease virus* (SPDV), is a widespread pathogen causing pancreas disease (PD) in European aquaculture of Atlantic salmon (*Salmon salar)* and rainbow trout (*Oncorhyncus mykiss)*. The six subtypes of SAV (SAV1-6) are geographically separated. SAV1 is common in Ireland and Scotland, SAV2 in rainbow trout reared in freshwater in continental Europe (1), but also in salmonids in sea cages in England, Scotland and Norway (2), and SAV3 is common in Norway where the disease has been recognized since 1989 (3). SAV4, 5 and 6 are occasionally detected in the British Isles (4). The subtype classification is based on the nucleotide sequence encoding the SAV proteins nsP3 and E2 (5). There is a general cross-neutralization between antibodies produced against all subtypes except SAV6, which represents a different serotype (6). SAV-infected fish demonstrate reduced appetite, aberrant swimming, fecal casts and lethargy, and the population has increased mortality. Subclinical infection can also occur (7). Histopathological changes, seen as degeneration and necrosis, are present in pancreas, heart and skeletal muscle. The main route of SAV transmission between fish is through shed virus in water, and between farms via water and well boat transport unless proper biosafety measures are implemented (8-10). The presence of SAV is monitored in marine aquaculture of salmonids in Norway by monthly RT-qPCR testing of farms. This is a costly process, and another reliable method to screen populations for SAV exposure could be an alternative or supplement.

SAV is an enveloped virus with a capped, polyadenylated, single-stranded RNA genome with positive polarity. The genome is 11.9 kb and consists of two large open reading frames (ORFs). The ORF1 encodes four non-structural proteins (nsP1-4), and the ORF2 encodes five structural proteins; the capsid, E3, E2, 6K and E1 proteins. The virion has an icosahedral nucleocapsid that encloses the genome, and is surrounded by a lipid envelope derived from the host cell (11). The major spikes embedded in the lipid envelope consist of trimers of E2 and E1 glycoprotein heterodimers. Both E1 and E2 are vital for viral entry, with E2 responsible for receptor recognition and binding, and E1 involved in host cell membrane penetration. During SAV replication, the non-structural region is directly translated into the polyprotein P1234, which is then cleaved into a replicase complex of nsP123 and nsP4. The nsPs, possible together with host proteins, will function as a polymerase complex that replicates the viral genome and transcribes the second ORF,

the subgenomic mRNA, which is translated into the structural polyprotein capsid-p62-6K-E1. Following its translation, the capsid is immediately autoproteolytically cleaved from the structural polyprotein and complexes with the genomic mRNAs to form the nucleocapsid of the new virus particles. The remaining structural polyprotein p62-6K-E1 translocate to the endoplasmatic reticulum, where E1 and E2 undergo proteolytic cleavage, glycosylation and maturation before travelling through the Golgi apparatus to the cell membrane for formation of progeny virus particles and budding (12).

According to the OIE Manual of Diagnostic Tests for Aquatic Animals 2019, a suspected case of infection with SAV is defined as the presence of one of the following: clinical signs, gross or microscopic pathologic lesions consistent with the disease, detection of antibodies against SAV, the detection of SAV, or contact with infected or suspected cases. Evidence for the presence of SAV is defined as confirmed when at least two independent laboratory tests have detected SAV (RT-qPCR or cell culture), antibodies against SAV (virus neutralization assay) or histopathological findings consistent with PD in the same fish. RT-qPCR is the OIE recommended test to be used in surveillance of SAV. Currently, all marine production sites in Norway sample hearts from 20 fish monthly for RT-qPCR testing (13). To be at least 95% sure to detect at least one SAV-positive fish in 20 random samples, the prevalence or infection rate must be at least 15 %.

Neutralizing antibodies are detected in SAV infected fish from around two weeks after infection (14, 15). In field trials, neutralizing antibodies against SAV have been shown to persist in a fish population for as long as 14 months (16). While RT-qPCR of heart tissue and histopathology are used to diagnose PD in Norway, neutralization assays are in use for routine diagnostics and to study epidemiology in United Kingdom and Ireland (17-20). While RT-qPCR is well suited at detecting early infection, this and other methods targeting serum antibody responses may have an advantage for diagnostic use in the later period of an infection, in subclinical cases and in cases with RT-qPCR results in the borderline between positive and negative. A longitudinal study of two outbreaks of PD caused by SAV1 indicated that a neutralization test was more sensitive and could detect positive fish for longer than RT-qPCR (16). Positive neutralization tests have also been found at slaughter from fish from farms deemed free of SAV through the whole sea water phase by the monthly RT-qPCR testing in Norway (21). Serological methods can also be used for

screening, for viral surveillance, to demonstrate freedom from disease, for prevalence studies in populations and for identification of reservoirs.

The virus neutralization assays are based on the ability of plasma or serum to inhibit virus replication in cell culture after pre-treatment of virus with the samples of interest. The method requires skilled personnel and is time consuming, as the incubation time for virus growth in cell culture is at least three days (18). An immunohistochemical method for SAV detection has been published (22), but could only detect virus in samples from the acute stages. Enzyme-linked immunosorbent assays (ELISAs) using recombinant E1 or E2 proteins as antigens have been used in research to detect antibodies against SAV (23, 24), but have not been in use for field diagnostics. Bead-based immunoassays, such as the xMAP assay, can be used to simultaneously detect antibodies against several pathogens, and we have previously used the method to detect antibodies against *Piscine orthoreovirus* (PRV) (25, 26). Such assays have been used to detect antibodies in mammals for almost two decades (27, 28). The analysis can be done in a few hours. It requires only a few microliters of sample material, making the assay suitable for non-lethal sampling. As high background binding and high levels of non-specific antibodies are found in plasma from Atlantic salmon, especially when collected in the field, a functional serological assay requires antigens that primarily bind specific antibodies. We have here tested the xMAP method with a bacterially expressed recombinant SAV3 E2 protein, whole SAV3 particles and disrupted SAV3 particles as source of antigens. The method was tested on plasma from an experimental SAV2 and SAV3 infection study and field samples from a PD outbreak caused by SAV2. We also compared the method with a virus neutralization assay.

MATERIALS AND METHODS

Cells

Chinook salmon embryo cells, (CHSE-214) (RRID:CVCL_2780), were grown at 20°C in Leibovitz (L15) medium supplemented with 10% heat inactivated foetal bovine serum (FBS), L-glutamine (2 mM), 2-mercaptoethanol (40 µM) and gentamicin-sulphate (50 µg/ml) (all from Life technologies, Paisley, Scotland, UK). Another CHSE cell line (CHSE-ppG) was kindly supplied by Bertrand Collet (INRA-French National Institute for Agricultural Research). These cells had been genetically engineered to contain the plasmid pcDNA3.1-HygmEGFP-pp4640 stably

expressing a SAV-1 structural polyprotein sequence (acc no. JX163854) with an N-terminal EFGP marker. The CHSE-ppG cells were grown in Eagle's Minimum Essential Medium (EMEM) at 20°C supplemented with 10% heat inactivated fetal bovine serum (FBS), 2-mercapethanol (40 µM) (all from Life technologies) and hygromycin (30 µg/ml) (ThermoFisher Scientific).

Virus propagation

CHSE-ppG cells were used to propagate the SAVH20/03 strain (acc. no. DQ149204). The cells were grown in 162 cm^2 Costar flasks (Corning Incorporated, Corning, NY, USA) for 48 hours prior to infection. The medium was removed, and cells washed twice with PBS before adding 5 ml virus suspension ($3x10^7$ $TCID_{50}$/ml) diluted 1:1000 in non-supplemented L15 medium and incubated for one hour at 15°C. This was followed by addition of 35 ml L-15 medium supplemented with 2% foetal calf serum (FCS), 2-mercaptoethanol (40 µM) and hygromycin (30 µg/ml) and continued incubation at 15°C for 10 days. Flasks were then transferred directly to -80°C. Uninfected CHSE-214 were used as negative control.

Concentration of SAV particles

SAV particles were concentrated by pelleting, using ultracentrifugation following a protocol developed for Semliki forest virus (29), with modifications. The samples were kept on ice throughout the entire protocol and cell lysis was performed using the freeze-thaw method on ten 162 cm^2 flasks containing infected CHSE-ppG and non-infected CHSE-214 cells. The CHSE-214 cell lysates were used as a background control. The lysed cell suspensions were then transferred to 50 ml centrifuge tubes (Corning Incorporated, Corning, NY, USA) and centrifuged at 10000g for 20 min at 4°C. The supernatants were transferred to 94 ml polyallomer centrifuge tubes, and the tubes sealed with aluminium caps (Beckman-Coulter, Brea, CA, USA). Ultracentrifugation was performed at 100000g for 150 min at 4°C using a fixed angle Beckman Type 45 TI rotor in an Optima LE 80K Ultracentrifuge (Beckman-Coulter, Brea, CA, USA). The top aqueous phase was removed, and the pellet suspended in 1 ml PBS and incubated overnight at 4°C with 250 rpm rotation. The suspensions were then stored at 4°C until further use.

RNA extraction and RT-qPCR

Quantitative real-time PCR was performed to confirm the presence of virus and to measure the genome copy number (GCN) ml^{-1} in the pellet. Extraction of viral RNA was performed using the QIAamp viral RNA mini kit (Qiagen, Venlo, Netherlands) following the protocol of the

manufacturer. Elution of RNA was performed with a 40 µl volume of elution buffer from the same kit. The RT-qPCR was performed using the Qiagen One-step RT-PCR kit and 3 µl eluted RNA in each reaction. Primers and probe, targeting a highly conserved region of the SAV nsp1 coding sequence, have previously been described (30). The conditions used in each reaction were 400 nM primer, 300 nM probe, 0.5 µl dNTP mix, 0.32 MgCl, 2.5 µl OneStep RT-PCR buffer, 0.5 µl OneStep enzyme mix and 2.3 µl RNase-free water in a total volume of 12.5 µl. For the generation of a SAV standard curve, 10-fold dilutions of a plasmid containing the complete SAV3 genome, the lowest concentration representing one plasmid copy and highest 10^9 copies, were run in three parallels together with the viral samples. The cycling parameters used were at 50°C/30 min and 95°C/15 min, followed by 40 cycles of 95°C/15 s, 60°C/1 min in an AriaMx (Agilent, Santa Clara, CA, USA). The presence of SAV in the pellet was confirmed with a C_t of 7.5. The standard curve slope, regression coefficient and amplification efficiency were -3.45, 0.999 and 94.3, respectively (**Suppl. Figure 1**). This indicates that the standard curve was near optimal and can be used for absolute quantitation. By extrapolation we get a GCN of 1.4×10^{11} ml^{-1}.

End-point dilution assay

$TCID_{50}$ assay was done to determine the amount of infective units per ml (IU ml^{-1}) in the pelleted virus. Chum salmon heart-1 cells (CHH-1) (RRID:CVCL_4143) was used for this purpose. Serial 10-fold dilutions were made of viral pellet suspension, and 50 µl of each dilution was added in eight parallels onto a 96-well plate containing a confluent monolayer. The infection protocol was as described above. The plates were incubated for ten days for the appearance of eventual cytopathic effects. CHH-1 cells were fixated and stained using an intracellular Fixation and Permabilization buffer (eBioscience, San Diego, CA, USA) and washed in Dulbecco's PBS (DPBS) with sodium azide. Cells were incubated with 17H23 anti-SAV E2 primary mouse antibody (31) followed by Alexa Fluor® 488 anti-mouse secondary antibody from Molecular Probes (Life Technologies). Dilutions of primary (1:1000) and secondary (1:400) antibodies were performed with permeabilization buffer in accordance with the manufacturer's protocol. For nuclear staining, Hoechst trihydrochloride trihydrate (Life Technologies) was used. Number of positive wells was quantified using an Olympus IX81 inverted fluorescence microscope. The Spearman-Kärber algorithm was used for calculation of $TCID_{50}$ expressed in IU ml^{-1}. The $TCID_{50}$ of the pelleted virus suspension was 2.0×10^{10} IU ml^{-1}.

SAV-E2 antigen

The SAV E2 protein was custom made by the MRC PPU Reagents and Services, University of Dundee (https://mrcppureagents.dundee.ac.uk) using the following procedure. The full-length E2 coding sequence (acc.no. JQ799139), presented in the plasmid pGex-SAV3 E2, was expressed as a GST-fusion protein in the *E. coli* (BL21) by induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) (50 µM). Bacteria were cultured for 16 hours before harvesting by centrifugation at 4200 x g for 20 mins at 4 °C. The pellet was resuspended in ice cold *E. coli* lysis buffer (50 mM Tris/HCl pH7.5, 250 mM NaCl, 1% Triton-X, 0.1 % 2-mercaptoethanol, 1 mM Pefabloc (4-(2-aminoethyl)-benzene-sulfonyl fluoride) and 1 mM benzamidine). Bacteria were lysed using a cell disruptor and extracts clarified by centrifugation at 30 000 x g for 20 min at 4 °C. Proteins were then purified using Glutathione S-transferase Agarose using standard procedure. Upon elution of the protein with elution buffer (wash buffer + 20 mM glutathione pH 7.5), the fractions containing protein were pooled with end over end mixing. This was subjected to purification on a HiTrap Heparin Sepharose HP column (GE Healthcare) equilibrated in equilibration buffer (50 mM Tris/HCl pH7.5, 0.03% (v/v) Brij 35, 0.1 mM EGTA, 0.1 % 2-mercaptoethanol). A linear salt gradient with increasing concentration up to 1 M NaCl was applied to the column and SAV E2 containing fractions were pooled and dialyzed into storage buffer (50 mM Tris/HCl pH7.5, 150 mM NaCl, 270 mM sucrose, 0.03% (v/v) Brij 35, 0.1 mM EGTA, 0.1 % 2-mercaptoethanol). The pooled protein was incubated for 16 hours with GST-PreScission Protease at 4 °C and a heparin column was used to separate the cleaved GST, GST-PreScission Protease and the SAV E2. The expression and protein yield were analyzed by SDS PAGE and Coomassie Blue staining.

Experimental salmon for plasma samples, sample collection and infection

Plasma from seawater-adapted Atlantic salmon post-smolts was taken from a previously published challenge trial (32, 33). Only plasma from the SAV-infected control group from the former study (not co-infected with PRV) was used in the present work. The fish had been challenged as described (32); briefly, shedder fish were i.p. injected with SAV2 or SAV3 (cell culture medium at a concentration of 10^4 $TCID_{50}$/ ml) and introduced to naïve fish four days later. Six fish from each SAV subtype infection were sampled at each sampling point (1, 2, 3 and 6 wpc). Blood was collected from the caudal vein on heparinized vacutainer tubes. After centrifugation, plasma was

collected and stored at -80 °C. Much of the plasma from 6 wpc had been used for other purposes, and only two samples from each subtype were available for analysis with the xMAP assay.

As a control for unspecific binding, plasma from a PRV-1 trial described in Lund and co-workers (34) was used. These samples had previously been used to detect antibodies against PRV (26)

The trials were approved by the Norwegian Animal Research Authority and performed in accordance with the recommendations of the current animal welfare regulations as outlined in: FOR-1996-01-15-23 (Norway).

Field samples

The field samples originated from a clinical PD outbreak caused by SAV2, as described in Røsæg and co-workers (pen number 10) (35). In short, the sampled fish were 1-year-old smolt of AquaGen strain, vaccinated with Alpha Ject micro 6 by Pharmaq and sea transferred in July 2014. During the first fall, the site was diagnosed with heart and skeletal muscle inflammation (HSMI), and the first detection of SAV-RNA from surveillance samples at site level was in April 2015. The fish group was then sampled regularly at 2-3-week intervals from the first SAV-RNA detection until slaughter. Clinical PD was first reported in week 27 in 2015 (29 of July 2015), based on evaluation from the fish health service, the site manager and a coinciding drop in appetite. This was denoted as week 0 of PD. At week 0 the average fish weight was 2.1 kg, and the accumulative mortality was 10.7%. At slaughter, the accumulative mortality was 14.35 % and the average slaughter weight was 4.2 kg.

The samples included in this study were composed of nine sampling points from 3 weeks prior to 15 weeks post the onset of clinical PD. Blood samples from ten fish per sampling point were collected in EDTA vacuum tubes, kept refrigerated and centrifuged within 24 h. Plasma was collected and stored at -20°C. For detection of SAV-RNA, the heart apex was sampled, stabilized on RNAlater® and either held refrigerated for 36-48 h before an overnight shipment or frozen below -20 °C prior to shipment. The analyses were performed by PatoGen AS according to validated and accredited methods (ISO17025 standard). Samples with no detection of virus were assigned a Ct of 38 (cut-off value) to include them in the graphical presentation.

Antigen preparation and coating on beads

Three different mixtures with whole virus were made from virus suspension from CHSE-ppG cells and coated on beads: 1) diluted 1:1 with PBS (SAV1:1), 2) diluted 1:10 with PBS (SAV1:10), and 3) mixed 2:1 with 0,1% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) in PBS and kept for 1 h at room temperature before coating (SAV-TX). A control was made by mixing two parts PBS containing the pellet from the uninfected CHSE-214 cells with one part PBS containing 0,1% Triton X-100 and kept for 1 h at room temperature before coating on beads (CHSE control). 60 µl of E2 protein from SAV3 (0,2 mg/ml) was used per coupling reaction for beads with E2 (SAV-E2). All coating was done according to the producer's instructions and as previously described (25, 26) using MagPlex® Microspheres #12, #21, #26, #29, # 34, #46 and #64 (Luminex Corp., Austin, TX, USA), N-Hydroxysulfosuccinimide sodium salt (Sigma-Aldrich), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (Sigma-Aldrich) and Bio-Plex Amine Coupling Kit (Bio-Rad, Hercules, CA, USA). PRV µ1c and DNP-KLH were conjugated to the beads as described earlier (25, 36).

xMAP assay

Titrations of plasma was performed, and a 1:50 dilution was found to give the best discrimination between control fish and infected fish from an experimental challenge trial (data not shown). This dilution was used for all samples except for when SAV-E2 and DNP-KLH beads were analyzed. Here, a 1:400 dilution was used to stay below the upper limit of quantification. As previously described (26), plasma was heated at 48 °C for 20 min to minimize non-specific antibody binding. The immunoassay was performed as described earlier (25). In short, plasma was diluted 1:50 or 1:400 in PBS and incubated at room temperature for 30 min on a shaker with MagPlex® Microspheres coated with the different antigen mixtures in a Bio-Plex Pro™ Flat Bottom Plate (Bio-Rad). Each sample was incubated with mouse AntiSalmonid-Ig monoclonal antibody (clone IPA5F12) (Cedarlane, Burlington, Ontario, Canada) diluted 1:400, biotinylated goat AntiMouse IgG2a antibody (Southern Biotechnology Association, Birmingham, AL, USA) diluted 1:1000 and finally Streptavidin-PE (Invitrogen, Carlsbad, CA, USA) diluted 1:50. Each incubation was performed at room temperature on a shaker at 600 rpm for 30 min. Three washings were done between each incubation. Beads coated with control proteins ICP11, FP native and FP-LM previously described (26) were also used. Samples were read using a Bio-Plex 200 (Bio-Rad), with median fluorescence intensity (MFI) as quantitative readout. The results were analyzed with the

BioPlex Manager 6.1 (Bio-Rad). The DD-gate was set to 5000-25000, and between 55 and 208 beads from each bead number were read from each well. The reading was carried out using a low PMT target value.

Neutralization assay

Samples from the field outbreak were heat-treated (48°C for 20 min) and a virus neutralization assay was performed as described earlier (18, 33) with CHSE-214 cells and acetone fixation before antigen detection with immunofluorescence. Endpoint titration of samples was performed if virus neutralization was observed at a 1:20 dilution.

Statistics

Statistical analyses used were Mann-Whitney test to find significant increase in binding to SAV-TX, SAV 1:1 and SAV-E2 beads, linear regression to examine the correlation between MFI from the different beads and Spearman's correlation to assess the relationship between MFI from beads and neutralization data (GraphPad Prism 7 and JMP Pro 14).

RESULTS

To assess the xMAP assay under controlled experimental conditions, we tested Atlantic salmon plasma from an infection study with SAV2 and SAV3 (32, 33). Plasma from 6 wpc showed an increased IgM binding to beads coated with SAV whole virus mixtures (**Figure 1a**). The beads with 1:10 dilutions of virus (not shown) gave similar results, although slightly lower for all weeks, compared to 1:1 dilution. Beads with Triton-X-treated, disrupted SAV particles (SAV-TX) showed similar results from week 1 and 2, a wider individual variation in binding in week 3 and significantly higher binding (detected as MFI) in week 6. Beads coated with recombinant SAV-E2 protein showed an increased binding to antibodies in plasma samples from 6 wpc. Plasma from both SAV2- and SAV3-infected fish bound to all bead types in a similar manner. Control beads coated with CHSE pellet (CHSE control beads) showed consistently low binding to all tested plasma, with MFI values below 50 (**Figure 1b**).

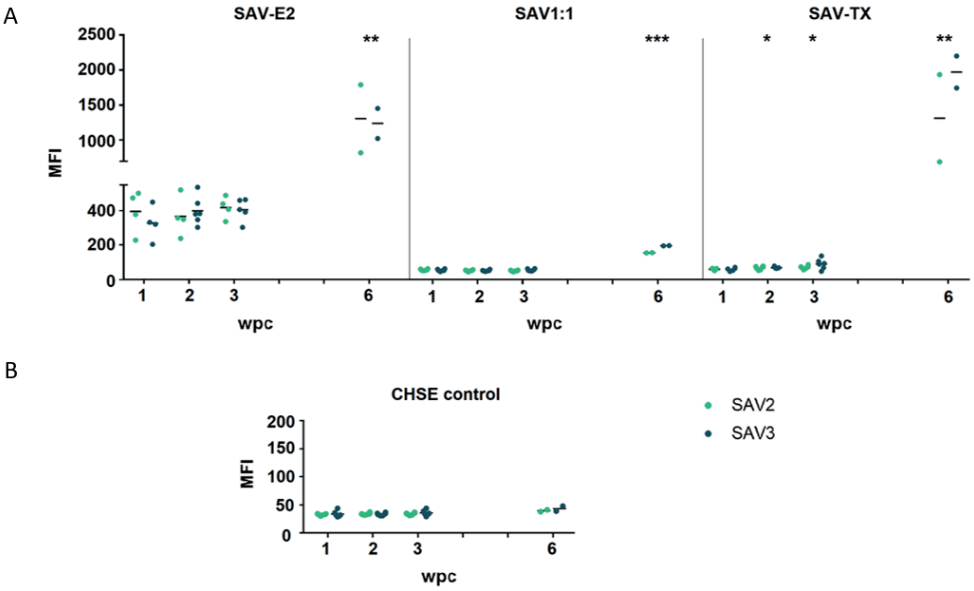


Figure 1. A) Comparison between SAV3 E2 protein (SAV-E2), whole SAV3 particles diluted 1:1 (SAV 1:1) and disrupted SAV3 particles (SAV-TX) coated on xMAP beads and used to test plasma from a SAV2/SAV3 cohabitant challenge for SAV-specific antibodies 1, 2, 3 and 6 weeks post challenge (wpc). B) Control beads coated with a lysate from the CHSE cells tested on the same plasma samples. MFI with mean values indicated by lines. Significant differences compared to 1 wpc are indicated by asterisks (Mann-Whitney test).

A few samples from SAV-injected shedders from the same challenge trial were also tested with the whole virus beads and the CHSE control beads. Again, the SAV-TX beads showed a higher MFI, rising to around 8000 for some fish, whereas binding to the CHSE control beads was low and did not increase (**Figure 2**). Since both the SAV1:1 and the SAV1:10 were inferior to the SAV-TX, only the latter was used as whole virus antigens in further analyses.

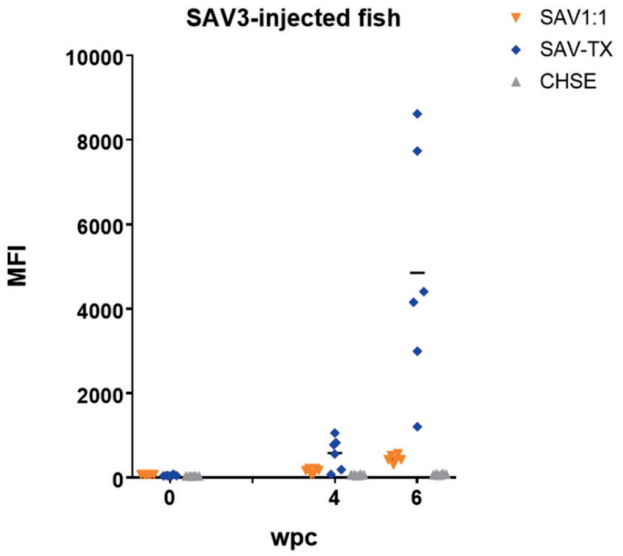


Figure 2. IgM detected in plasma from Atlantic salmon injected experimentally (i.p) with SAV3 using an xMAP assay with beads coated with whole SAV3 particles (SAV 1:1) and disrupted SAV3 particles (SAV-TX) as antigen and control beads (CHSE). MFI levels with mean.

To evaluate the specificity of the SAV antigens, the SAV-E2 and SAV-TX were tested on plasma from a PRV challenge trial together with other control antigens (**Figure 3**). We have previously showed that a PRV infection induces production of non-specific, possibly polyreactive antibodies (26), and found high background MFI from beads coated with the irrelevant proteins: lipid-modified ICP11 protein from shrimp white spot syndrome virus (ICP11), lipid-modified infectious salmon anemia virus fusion protein (FP-LM) and an unmodified infectious salmon anemia virus fusion protein (FP). In contrast to these proteins, SAV-TX did not show any binding of non-specific IgM. SAV-E2 however, showed a similar binding pattern as the irrelevant antigens.

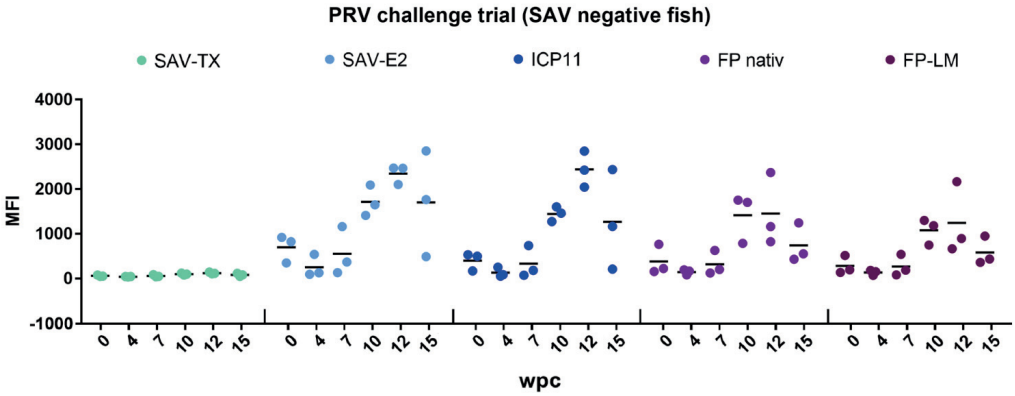


Figure 3. Background IgM binding to beads coated with SAV antigens or antigens from other irrelevant viruses measured in plasma from an experimental PRV challenge trial. MFI with mean.

To assess the performance of these assays under more realistic conditions, samples from a field PD outbreak were analyzed using SAV-E2, SAV-TX and CHSE control beads as well as PRV1-µ1c beads used previously to detect PRV specific antibodies (25). Plasma samples from 3 and 1 weeks before, and 2, 4, 6, 8, 10, 13 and 15 weeks after the defined start of the outbreak were analyzed. RT-qPCR showed viral RNA presence in fish from week 2 after the outbreak (**Figure 4a**). The binding of plasma antibodies to SAV-TX beads started to increase from around two weeks after the PD outbreak, except for one fish (of the 10 tested) that was positive in the week before the outbreak (**Figure 4b**). SAV-TX binding reached a maximum at around 8 weeks after the outbreak. At 13 and 15 weeks there were still many positive fish, while at these time points most of the samples were negative for SAV RNA by RT-qPCR (**Figure 4**).

We detected antibodies binding to SAV-E2 beads from all time points, including before the outbreak. There was an increase in mean values from before the outbreak to 10 weeks after, similar to what was seen with SAV-TX, but the individual variations at each time point were very high (up to >10 000 MFI) even before the outbreak (**Figure 4b**), and the increase was only statistically significant in week 10 and 13.

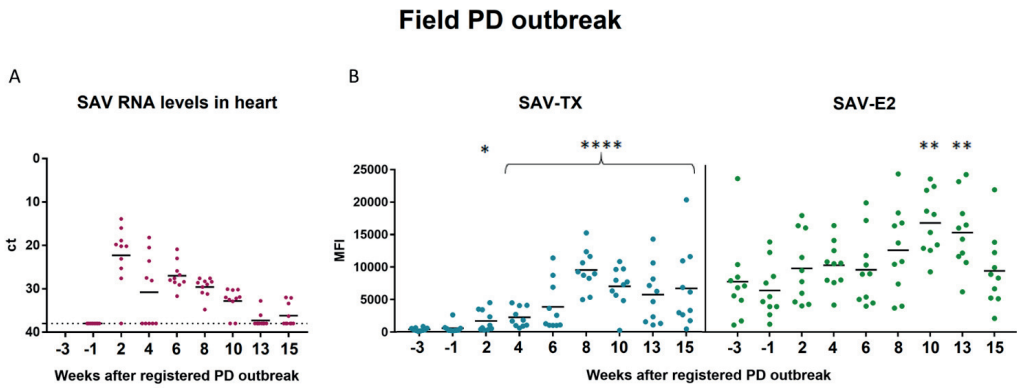


Figure 4. Analysis of samples from a field outbreak of pancreas disease caused by SAV2. A) SAV RNA levels in hearts. Mean values indicated with lines. B) Levels of antibody binding to SAV-TX and SAV-E2 coated beads from plasma from the same outbreak. Mean values indicated with lines. Significant differences compared to three weeks before the outbreak are indicated by asterisks (Mann-Whitney test).

After the outbreak, all samples except four had either neutralizing antibodies or viremia (**Figure 5).** The presence of neutralizing antibodies could not be determined in viremic samples due to interference of the virus with the test principle. Neutralization titers and binding to SAV-TX followed roughly the same pattern, with antibody levels rising from around two weeks after the outbreak and staying elevated until the end of the sampling period, with some individuals deviating from this. Strong to moderate correlation between neutralization titers and MFI values of SAV-TX and SAV-E2 was found. A Spearman's correlation shows $\rho = 0{,}73$ ($p<0{,}0001$) and $\rho = 0{,}43$ ($p=0{,}0001$) for SAV-TX and SAV-E2, respectively (**Suppl. Figure 2**). There were, however, some individuals with high levels of neutralizing antibodies and very low levels of binding to SAV-TX and some negative for neutralizing antibodies with increased binding to SAV-TX. Weak correlations were found between neutralizing antibodies and binding to the other beads in the xMAP analysis (**Suppl. Figure 3**).

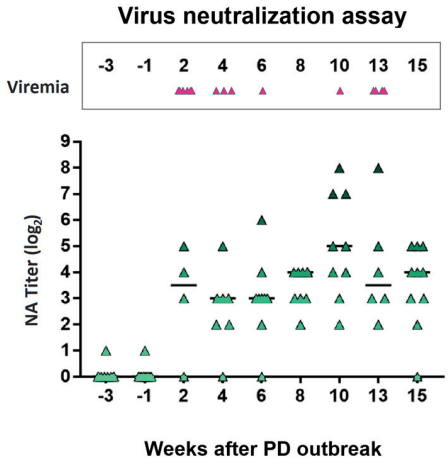


Figure 5. Titer of neutralizing antibodies from samples from the field PD outbreak with median values indicated by lines. Neutralizing activity could not be determined in samples with viremia (in pink).

Many of the field sample plasma from all time points contained antibodies binding to CHSE control beads. The levels were higher than in the challenge trial, but unlike the SAV-E2 results there were no clear increase over this time period (**Figure 6a**). A high degree of binding was also found to PRVμ1c and DNP-KLH coated beads (**Figure 6b and 6c**).

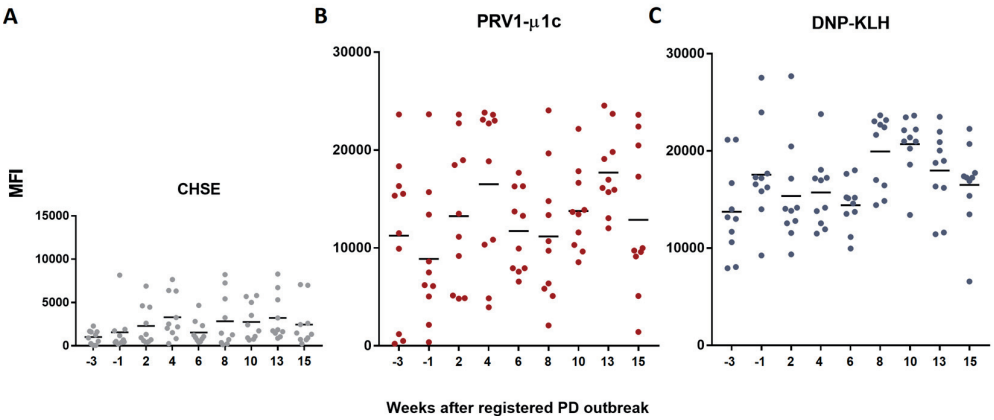


Figure 6. Antibodies binding to CHSE control (A), PRV1-μ1c (B) and DNP-KLH (C) beads from plasma from the PD outbreak. MFI with mean.

By regression analysis, we found a correlation between binding to CHSE control and SAV-E2 coated beads (**Figure 7**). The binding to SAV-TX coated beads was not correlated to binding to

the CHSE control beads (**Figure 7**). Binding to DNP-KLH had a low correlation (r^2 = 0,21, p>0,0001) with SAV-E2 and even lower or no correlation with the other beads (**Suppl. Figure 4**).

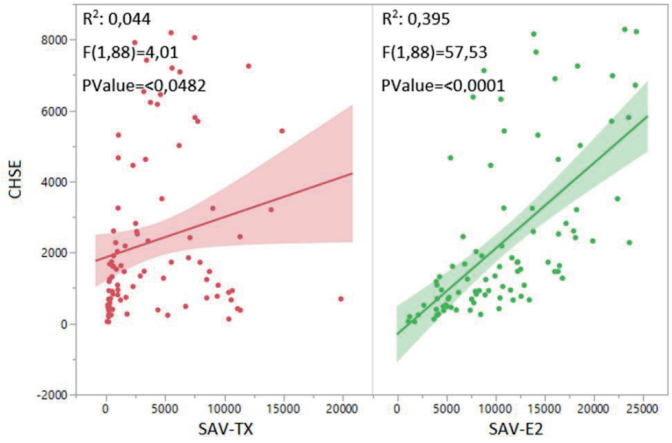


Figure 7. Correlation between CHSE control and SAV-TX and SAV-E2 beads.

DISCUSSION

In this study we showed that a bead-based immunoassay with disrupted SAV particles was well suited to detect SAV specific antibodies in plasma samples from both experimental infections and field outbreaks. Our results show that antibodies could be detected from between 3 and 6 wpc in cohabitant fish and from around 4 wpc in virus-injected fish. In the field outbreak of PD, a significant increase in SAV-specific antibodies was detected from two weeks after the clinical outbreak. One fish also had an increased antibody level one week before the outbreak, probably reflecting that the start of the outbreak occurred before infection was detected, and that a low prevalence of infected fish occurs for a period in the early stages of an outbreak. The level of antibodies in most samples remained elevated at the last sample point at 15 weeks after the outbreak, when only one sample had a lower MFI than the highest MFI at three weeks before the outbreak. At this sampling point 70% of the samples were found negative by RT-qPCR.

The stably transfected CHSE-ppG cells express a plasmid that contains the SAV-1 structural polyprotein with an N-terminal EFGP tag. No viral-like particles are produced by these cells, most likely because the EGFP tag will interfere with the capsid construction given that it is

approximately the same size as the capsid (30 kDa). Since the capsid is immediately cleaved from the structural polyprotein after translation in the cytoplasm, the folding and structural conformation of E2 and E1 should not be affected, as this takes place in the endoplasmatic reticulum (12). When the CHSE-ppG cells are infected with SAV-3 H20/03 they yield a viral titer of 3.4 x 10^9 $TCID_{50}$/mL, compared to CHSE-214 cells that typically produce a titer of approximately 10^6 in our laboratory. The limiting factors for SAV production in CHSE cells are unknown, but the increased access to E1 or E2 could be a positive factor in the production of virus particles, and an explanation why the CHSE-ppG produced more virus. The CHSE-ppG cells express SAV-1 structural polyprotein while the infectious virus added was a SAV3 subtype and it is not known if the virus particles produced in CHSE-ppG were SAV1/3 hybrids. However, due to the cross-neutralization between the SAV1-5 subtypes this would probably not influence the binding of specific antibodies against SAV3 or SAV2 (6). The CHSE-ppG cells were therefor preferred for virus propagation to ensure sufficient amount of antigen for the xMAP assay.

Alphaviruses can be difficult to purify due to the fragility of the virion envelope (37). A study comparing methods of density-gradient centrifugation of the alphavirus Semliki forest virus resulted in virus recovery of 6-40% (38). Due to this great loss of virus particles we chose direct pelleting by ultracentrifugation as a method of concentration of SAV particles rather than purification through a gradient cushion. A higher concentration of virus particles was considered of greater importance than having a pure sample since the xMAP assay was run with a negative control of uninfected CHSE-214 cells also pelleted by ultracentrifugation. The obtained value for GCN ml^{-1} was one-fold higher than those calculated for IU ml^{-1}. A higher GCN ml^{-1} is to be expected since this will include RNA from virus without considering their infectivity. Real-time PCR assay will over-estimate the number of particles since viral RNA-target is also detected from free viral RNA from damaged cells and replication intermediates. At the same time a $TCID_{50}$ assay will under-estimate the amount of virus particles since defective virus (non-infectious) are not evaluated. We therefore conclude that the number of viral particles in the pellet suspension lies somewhere in between these two estimates (2.0 x 10^{10} - 1.4 x 10^{11}).

The non-ionic detergent Triton-X was used to disrupt the virus particles. This has been successfully used previously to disrupt virus particles for coating on beads for immunoassays (39, 40). Triton-X solubilize lipid-free membrane proteins without disruption of the native structure and biological

function. It has been used to isolate E1 and E2 of the alphavirus Western equine encephalitis virus but could not alone separate E1 and E2. Furthermore, the capsid was not affected by the treatment (41), where the concentration of Triton-X was higher than that used by us in the present study. Therefore, it is likely that mainly intact E1-E2 heterodimers and virus capsids were coated on the SAV-TX beads.

The correlation between binding of antibodies to SAV-TX beads and neutralization titers was strong. There were, however, some individuals with high levels of neutralizing antibodies and very low levels of binding to SAV-TX beads and some that were negative for neutralizing antibodies that still had increased level of antibodies binding to SAV-TX. This indicates that SAV-specific antibodies and SAV-neutralizing antibodies are different although partly overlapping variables and that in some individuals, the humoral immune response did not target the neutralizing epitopes. Because of these large individual variations and partly unresolved specificities, serological testing of salmon for SAV infection should primarily be used for groups of fish and not individuals.

There was only a moderate correlation between SAV-E2-binding and neutralizing antibodies. In alphaviruses infecting mammals, monoclonal antibodies against E2 are more often neutralizing than monoclonal antibodies against E1 (42, 43). However, when studying both IgM and IgG produced after natural infection with the alphavirus *Chikungunya virus* in humans, Chua and co-workers found that while neutralizing IgG bound to epitopes on E2, neutralizing IgM bound to specific conformation-dependent epitopes on the E1-E2 heterodimers (44). This could also be the case in salmon as IgM is the dominating antibody. Unlike neutralizing antibodies that bind to a limited number of epitopes on the virus, the non-neutralizing specific antibodies can bind to any available epitope. While neutralizing antibodies can work in several different ways to block infectivity, non-neutralizing antibodies can control infection by antibody-dependent cellular toxicity, complement-mediated lysis with the formation of a membrane attack complex, and Fc receptor-mediated phagocytosis. The relevance of the neutralizing and the specific antibodies in relation to protection against PD requires further research.

It has earlier been shown that plasma from PRV infected fish can have a neutralizing effect on SAV. The neutralizing effect was lost in the majority but not all of the samples after heat-treatment (48°C for 20 min) (33). This could have been caused by polyreactive antibodies or by other factors present in the samples. When testing SAV-TX beads on plasma from PRV-infected fish, no

increase in antibody binding were seen from any of the samples (**Figure 3**). This suggests that although there is a high level of non-specific antibodies present in these samples, the SAV-TX lysate only binds highly specific antibodies against SAV. The SAV-E2 antigen appeared to be a good candidate for SAV diagnostic based on the results from the SAV challenge trial. However, when tested on plasma from PRV-infected (SAV-free) fish we found a high degree of binding to SAV-E2, and we also found a high background binding in samples from the field outbreak.

When looking at binding to PRVμ1c beads, we found very high values at all time points (**Figure 6b**). This is not unexpected since PRV is a ubiquitous and persistent virus (45, 46), and since HSMI was diagnosed around six months prior to the PD outbreak. The CHSE control beads also resulted in variable and high IgM binding in the field samples, compared to very low values in the challenge trial. When using regression analysis, we found that a large part of the increase in CHSE control bead binding occurred together with an increase in PRV-μ1c binding (**Suppl. Figure 5**), but not along with SAV-TX binding (**Figure 7**). SAV-E2 binding was also correlated with CHSE control bead binding (**Figure 7**). High levels of polyreactive antibodies are found in PRV-infected fish (26), and can also be increased after vaccination (36), after exposure to antigens in the environment or infection with other pathogens (47). Taken together, this indicates that binding to SAV-E2 and CHSE control beads to a large degree represent unspecific binding by non-specific or polyreactive antibodies. However, the correlation between SAV-E2 and the hapten-carrier antigen DNP-KLH, often used to measure polyreactive antibodies, was low. Polyreactive antibodies are often self-reactive (48), and as CHSE cells are Chinook salmon embryo cells, the observed binding may possibly be constituted of self-reactive Atlantic salmon antibodies. The fish from the field outbreak were vaccinated with an inactivated vaccine containing five bacteria and infectious pancreas necrosis virus. The binding to SAV-E2 seen in the field samples is likely a mixture of specific antibodies against SAV, also seen in the challenge trial, antibodies against other antigens the fish has been exposed to and polyreactive antibodies.

Many fish are now vaccinated against PD, and the xMAP assay should be evaluated for use in PD-vaccinated fish. Chang and co-workers studied the antibody production and protection after vaccination with a commercially available inactivated vaccine and two DNA vaccines against SAV (24). The inactivated vaccine provided reduction of viral levels in serum and partial protection against PD, but neither antibodies against E2 nor neutralizing antibodies. The DNA

vaccine encoding only the E2 protein provided no antibodies against E2, no neutralizing antibodies, no reduction in pathology and no reduction of virus level in serum. The DNA vaccine encoding the whole structural polyprotein of SAV provided reduction of virus levels in plasma, antibodies against E2, virus neutralizing activity and full protection against pathological changes typical of PD. In another vaccine challenge comparing DNA vaccines and subunit vaccines with E1 or E2 and an inactivated vaccine, levels of antibodies against E2 did not correlate with level of neutralizing antibodies or reduction of mortality (23). The E2 protein is dependent on co-expression with E1 and lower temperatures for correct folding and for translocation and presentation on the cell surface (49). Thus, the recombinant E2 used here may not possess antigenic properties equivalent to the native protein, and thus not bind a large proportion of the SAV-specific antibodies. However, non-specific, possibly polyreactive antibodies produced after PRV infection may bind to this antigen. As PRV is ubiquitous in farmed fish in Norway, our results are not consistent with reliable use of this antigen on field material.

The Norwegian coast line is divided into different zones by fish health authorities regarding management of PD, with the aim to control or eradicate SAV infection from farmed fish. Serological methods can have advantages for surveillance compared to the virus detection by RT-qPCR that is currently used, as serology may reveal former infection by and previous contact with the virus longer than the virus itself can be detected in the fish (16, 50). The xMAP method requires small amounts of sample material and is time saving compared to virus neutralization assays and RT-qPCR. The method would also be suitable for non-lethal testing and for testing of many fish in a surveillance program, especially to document freedom from SAV outside of endemic zones. Sampling could be done when the fish is handled for lice counting or other events or at slaughter, and plasma from several fish could be pooled. When using pooled samples, more fish can be tested, and the distribution of SAV can be better monitored, even when the prevalence is low. More testing, including on subclinical SAV infections, later than 15 weeks after the start of a PD outbreak, in pooled samples and on SAV-vaccinated fish, should be done to confirm that this method is at least as sensitive and specific as neutralization assays and to examine the effects on SAV infection by the production of both neutralizing and non-neutralizing antibodies. A challenge with serological tests in Atlantic salmon has been to find good antigens that can bind specific antibodies with limited background binding. The present study indicates that Triton-X-treated whole SAV particles may indeed fulfill this goal.

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SUPPLEMENTARY FILES

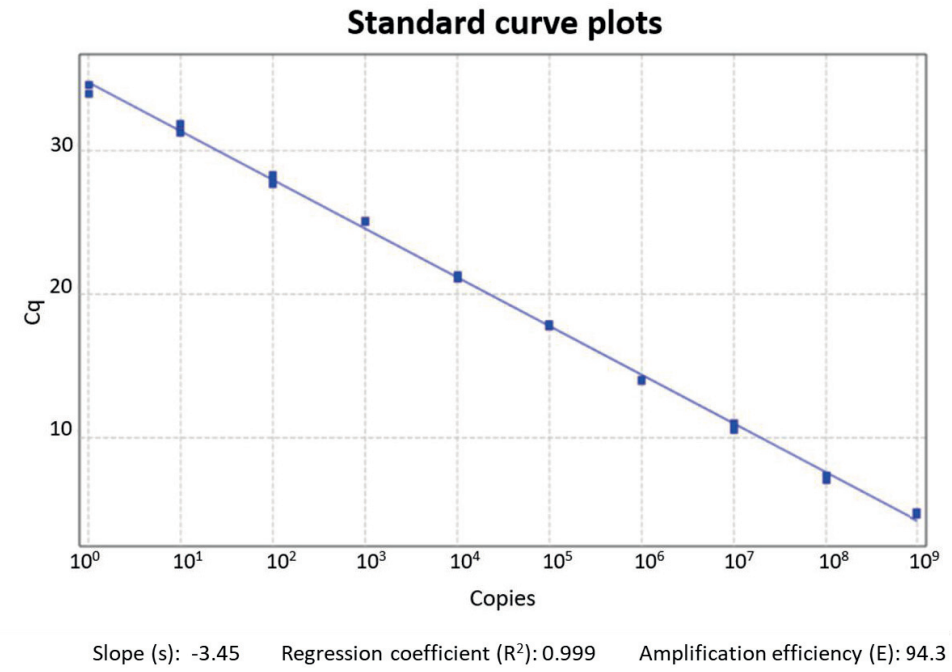

Figure S1. Ten-fold serial dilutions of plasmid expressing SAV-3 genome, and Ct-values (Cq) for each triplicate plotted against number of copies in each reaction.

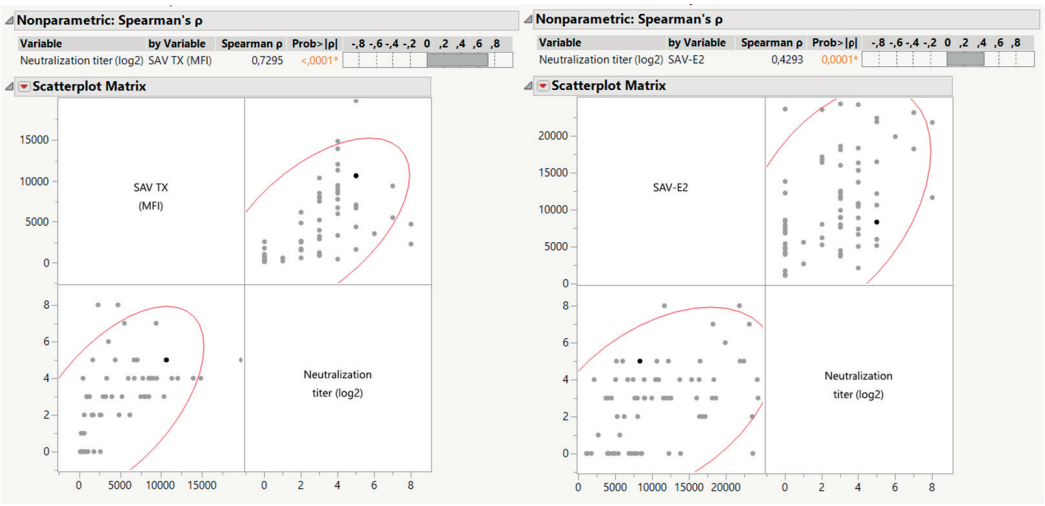

Figure S2. Correlation between neutralization titers and binding to SAV-TX and SAV-E2 beads

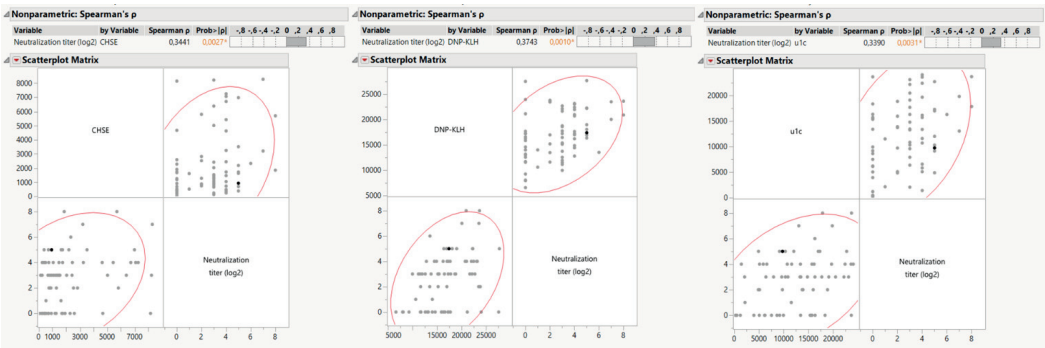

Figure S3. Correlation between neutralization titers and binding to CHSE, DNP-KLH and µ1c beads

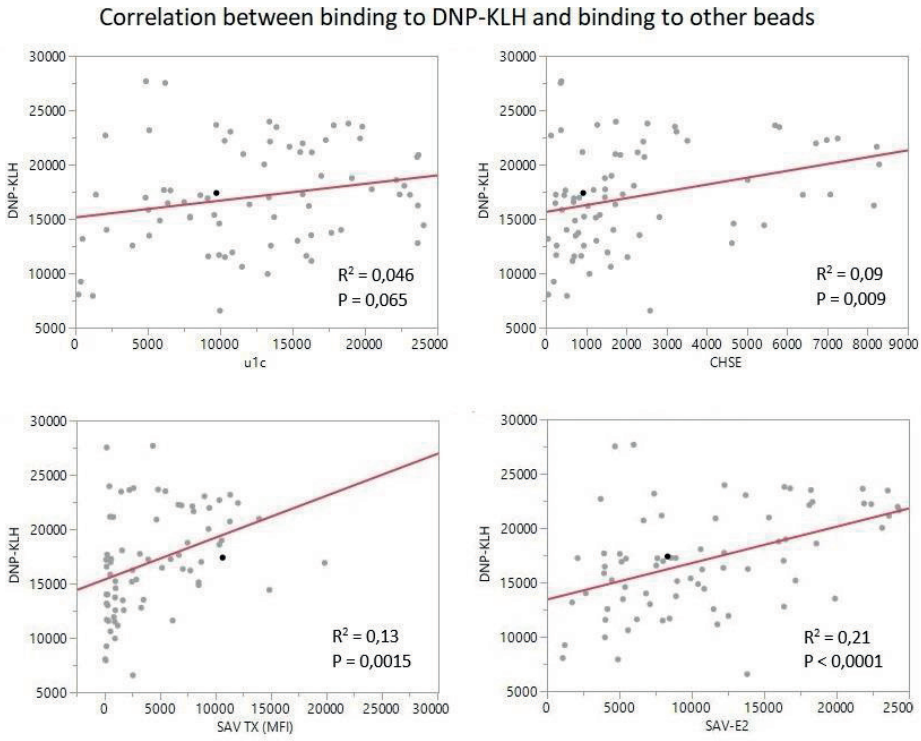

Figure S4. Correlation between binding to DNP-KLH and binding to µ1c, CHSE, SAV-TX and SASV-E2 beads

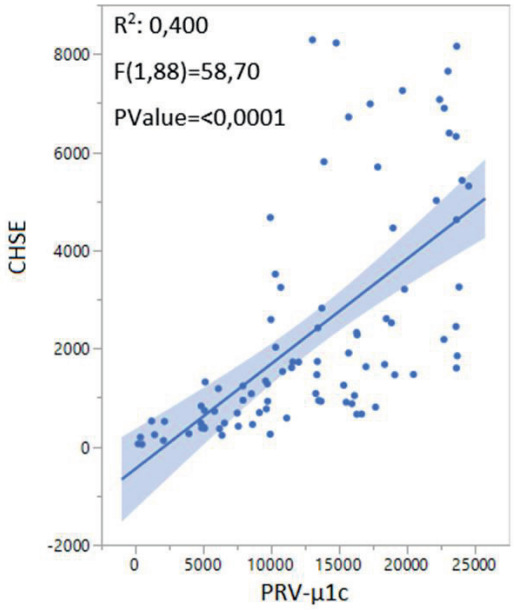

Figure S5. Correlation between binding to CHSE and PRV-µ1c beads

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Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

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