# Adaptive immune responses in Atlantic salmon (*Salmo salar* L.) after vaccination with bacterins of *Pasteurella atlantica*

Vemund Magne Holstad


Master of Science in Aquamedicine

Department of Biological Sciences

University of Bergen, Norway

June 2022

2022

Adaptive immune responses in Atlantic salmon (*Salmo salar* L.) after vaccination with bacterins of *Pasteurella atlantica*

University of Bergen

http://bora.uib.no/

# Acknowledgements

Firstly, I would like to thank my main supervisor associate Professor Anita Rønneseth for her work and time put into this master thesis. You have been much more helpful than I would have hoped a supervisor could be. Thank you for your guidance.

Secondly, I would like to thank my co-supervisors Dr. Cyril Frantzen, associate Professor Gyri Teien Haugland and Professor Heidrun Wergeland. Cyril, thank you for your help with the phylogenomic analysis and proof reding of this mater thesis. Gyri and Heidrun, thank you for your sharing of knowledge and help at the laboratory. I would also like to give a special thanks to Rebecca Marie Ellul and Harald Sæbø Lunde for your help at the laboratory and sharing of your knowledge. Thank you.

Furthermore, I would like to thank my fellow students and friends for making these five years unforgettable and fun. I would also like to thank my family for all your support. Lastly, I would like to thank my colleagues at Akvavet Gulen AS, Eide Fjordbruk AS and Gjølanger Settefisk AS for a magnificent work environment throughout these five years. Thank you all.

Bergen, June 2022

Vemund Magne Holstad

# List of abbreviations

| Abbreviation | Full name |
|---|---|
| Ab | Antibody |
| Ag | Antigen |
| BA | Blood agar |
| BcR | B cell Receptor |
| CD4+ | Cluster of Differentiation 4 positive |
| CD8+ | Cluster of Differentiation 8 positive |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FAB | Fragment Antigen-Binding |
| FC | Fragment Crystallizable |
| FCS | Fetal Calf Serum |
| g | Gram |
| *g* | Gravitational force |
| Ig | Immunoglobin |
| IPN | Infectious Pancreatic Necrosis |
| IPNV | Infectious Pancreatic Necrosis Virus |
| kDa | Kilodalton |
| L | Liter |
| M | Molar |
| mg | Milligram |
| MHC | Major histocompatibility complex |
| ml | Milliliter |
| ng | Nanogram |
| nm | Nanometer |
| NVI | Norwegian Veterinary Institute |
| o/n | Overnight |
| OD | Optical Density |
| OMP | Outer Membrane Protein |
| p | Probability value |
| PBS | Phosphate Buffered Saline |
| PBS-Tween | Phosphate Buffered Saline with 0.05% Tween |
| PCR | Polymerase Chain Reaction |
| PD | Pancreas Disease |

| | |
|---|---|
| pmol | Picomole |
| qPCR | quantitative Polymerase Chain Reaction |
| RPS | Relative Percentage Survival |
| Rt | Room temperature |
| SD | Standard Deviation |
| SDS-PAGE | Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis |
| SPDV | Salmon Pancreas Disease Virus |
| TBS | Tris-Buffered Saline |
| TcR | T cell Receptor |
| TSB | Tropic Soy Broth |
| TTBS | Tween Tris-Buffered Saline |
| WB | Western Blot |
| WBC | White blood cell |
| xg | Times gravity |
| µl | Microliter |

# Abstract

Outbreaks of pasteurellosis in Atlantic salmon (*Salmo salar* L.) have since 2018 become a major problem in the farming industry, and yet not commercially available vaccine have been developed. Little is currently known about the bacteria's surface proteins, immunogenic components, virulence factors, if it is a homogenous group or if there are differences between isolates of the group when it comes to expression of surface proteins after in vitro culturing. Also, little is known about how *Pasteurella atlantica* isolates from salmon and isolates from lumpsuckers (*Cyclopterus lumpus* L.) differ with respect to these factors.

In this thesis we vaccinated Atlantic salmon with three isolates of *P. atlantica* genomovar *salmonicida* isolated from commercially produced salmon. We studied the presence of specific antibodies in the sera post vaccination and tested for cross-reactivity across all three isolates, as well as one isolate isolated from lumpsucker. We also studied these isolates' protein profiles through silver stained SDS-PAGE gels and immunogenic proteins through western blotting. Lastly, a phylogenomic analysis was performed to study the relationship between the isolates, as well as two *Pasteurella skyensis* isolates.

We found that the salmon immune sera were highly cross-reactive across all salmon isolates, but only slightly cross-reactive to the lumpsucker isolate when analyzed by ELISA. We found that the salmon isolates were a homogenous group regarding their protein profile and immunogenic proteins and differed significantly from the lumpsucker isolate. Lastly, these differences and similarities are substantiated through the phylogenomic analysis, showing differences between the salmon isolates and the lumpsucker isolates, but that the *P. atlantica* isolates are more similar to each other than to the *P. skyensis* isolate.

These findings indicate that vaccines based on one isolate can be developed to protect salmon from pasteurellosis. These findings also indicate the differences between *P. atlantica* isolated from salmon and lumpsuckers and underlines that knowledge on genomovar *cyclopteri* is not necessarily transferable to genomovar *salmonicida*.

# Samandrag

Utbrot av pasteurellose hjå Atlanterhavslaks (*Salmo salar* L.) har sidan 2018 vore eit stort problem i oppdrettsnæringa, og framleis har inga kommersielt tilgjengeleg vaksine blitt utvikla. Vi veit lite om bakteriens overflateprotein, immunogene komponentar, virulensfaktorar, om det er ein homogen gruppe eller om det er forskjellar mellom bakterieisolata når det kjem til uttrykk av overflateprotein etter in vitro kultivering. Vi veit også lite om korleis *Pasteurella atlantica* isolat frå laks og isolat frå rognkjeks (*Cyclopterus lumpus* L.) skil seg frå kvarandre med tanke på desse faktorane.

I denne masteroppgåva har vi vaksinert Atlanterhavslaks med tre isolat av *P. atlantica* genomovar *salmonicida* isolert frå kommersielt produsert laks. Vi studerte om det var spesifikke antistoff i sera etter vaksinering og testa for kryss-reaktivitet på tvers av alle tre isolata, i tillegg til eit isolat isolert frå rognkjeks. Vi studerte også desse isolata sine proteinprofilar gjennom SDS-PAGE gelar og deira immunogene protein med western blotting. Til slutt vart ein fylogenetisk analyse gjennomført for å studere slektskapet mellom desse isolata, i tillegg til to *Pasteurella skyensis* isolat.

Vi fant ut at laksen sitt sera var svært kryss-reaktivt på tvers av alle lakseisolata, men berre litt kryss-reaktivt til rognkjeksisolatet når dei vart analysert med ELISA. Vi fant ut at lakseisolata er ein homogen gruppe når det kjem til deira proteinprofil og immunogene protein, og at dei skil seg signifikant frå rognkjeksisolata. Til slutt vart desse forskjellane og likskapane underbygga gjennom fylogenetiske analyser, som viser forskjellar mellom lakse- og rognkjeksisolata, men at *P. atlantica* isolata er meir lik kvarandre, enn med *P. skyensis* isolata.

Desse funna indikerer at ein vaksine basert på ein av isolata vil kunne beskytte laksen mot pasteurellose. Desse funna indikerer også at det er forskjellar på *P. atlantica* isolat frå laks og rognkjeks, og at kunnskap frå genomovar *cyclopteri* ikkje nødvendigvis er overførbart til genomovar *salmonicida*.

# Table of Contents

# List of Figures

# List of tables

# 1 – Introduction

In the year 2021, 45 Norwegian Atlantic salmon (*Salmo salar* L.) fish farms were diagnosed with pasteurellosis, and the disease was recognized as the 8$^{th}$ largest fish health problem in the industry according to a survey performed by the Norwegian Veterinary Institute (NVI) (Sommerset *et al.*, 2022). The Norwegian fish farming industry produces over 1.4 million metric tons of salmonids annually. An important factor for making this large-scale production possible is the availability of efficient vaccines. Each year 350 million salmon are vaccinated, and the vaccines protect against major bacterial and viral diseases. In Norway, the production of farmed salmon occurs nearly without any use of antibiotics, with only 605 kg of antibiotics having been prescribed in 2021 (Directorate of fisheries, 2021; Litleskare, 2022). Underlining that vaccination against bacterial diseases is key to keeping the usage of antibiotics low in the industry. Currently there are no commercially available vaccines against the emerging disease pasteurellosis in Atlantic salmon, making this a crucial issue both for ethical and economic reasons, and to reach the point where protective vaccines can be developed. Research is needed to gain more knowledge of the causative agent, *Pasteurella atlantica* genomovar *salmonicida*.

## 1.1 – *Pasteurella* sp. in Atlantic salmon

Pasteurellosis is the name of the disease caused by bacterial infections by bacteria belonging to the genus *Pasteurella*. The first detection of pasteurellosis in Norwegian farmed salmon was in 1989 and the disease later got the name “VarraccaIbmi”, meaning blood eye in the Sami language (Valheim *et al.*, 2000). Pasteurellosis had not been a significant problem in the Norwegian salmon farming industry, until 2018 (Sommerset *et al.*, 2021), when a new member of the *Pasteurella* species was isolated from diseased salmon. Incidences of pasteurellosis caused by this new species have been steadily increasing and have now spread along the Norwegian coastline. The NVI have suggested the working nomenclature *Pasteurella atlantica* genomovar *salmonicida*, until an official name for these isolates is published. In September 2020 *Pasteruella skyensis*, known to induce pasteurellosis in farmed salmon in Scotland, was detected in farmed Norwegian Atlantic salmon. However, this was limited to only one fish farm and has not yet caused significant problems.

*P. atlantica* genomovar *salmonicida* can be cultured from the kidney, heart, liver, spleen, and muscle abscess samples of infected salmon, and can be cultured on blood agar plates with 2% NaCl (Legård and Strøm, 2020). The colonies are slow growing, white/colorless, and about 1 mm in diameter. Alpha hemolysis on the blood agar plates may be present and seems to be temperature dependent. The bacteria are non-motile, rod shaped, gram negative and oxidase positive.

The main characteristics of the pathology described on fish from the outbreaks of pasteurellosis, or varraccalbmi, in the early 1990s was hemorrhagic and necrotizing inflammation in the eye, as well as deep dermal ulcerations and necrosis of the pseudobranch (Valheim *et al.*, 2000). In the outbreaks after 2018 caused by *P. atlantica* genomovar *salmonicida*, blood filled abscesses in skeletal and heart muscles are important characteristics, as well as inflammation in the pericardium, abdominal wall and pectoral fin basis (Legård and Strøm, 2020; Sommerset *et al.*, 2021). These characteristics, combined with an increase in mortality, have a huge impact on fish welfare and lead to major economic losses within the industry.

Pasteurellosis occur all year round, but is most frequently reported when the sea temperatures are between 7.9 °C and 18 °C (Legård and Strøm, 2020). Disease outbreak often occurs about 14 days post sea lice treatments. This may be due to stressors inflicted on the fish by the treatments, causing disease in asymptomatic carrier fish and/or poor biosecurity during treatments (Gismervik *et al.*, 2019; Nilsson *et al.*, 2019; Sommerset *et al.*, 2021).

## 1.2 – *Pasteurella* sp. in lumpsuckers

Whilst this master thesis mainly concerns *P. atlantica* genomovar *salmonicida* isolated from Atlantic salmon, infections of *Pasteurella* sp. in lumpsuckers (*Cyclopterus lumpus* L.) have been more common in Norwegian aquaculture prior to 2018, and thus our knowledge of *Pasteurella* sp. in lumpsuckers is greater (Legård and Strøm, 2020; Sandlund *et al.*, 2021; Sommerset *et al.*, 2021). Lumpsuckers have been used in Norwegian aquaculture as a biological prophylactical treatment against sea lice (*Lepeophtheirus salmonis*), as the lumpsucker eats the lice attached to the salmon's body.

The NVI has conducted whole genome sequencing and comparison of >80 isolates of *Pasteurella* sp. harvested from salmon and lumpsuckers in Norway and Scotland (Gulla *et al.*, 2020; Sommerset *et al.*, 2021). They found that all samples from Scottish salmon belonged to *P. skyensis*¸ and that almost all samples from Norwegian salmon were not *P. skyensis*. The *Pasteurella* sp. isolates causing pasteurellosis in Norwegian salmon and lumpsuckers were genetically distinct enough to be considered a separate species from *P. skyensis*. Furthermore, they were genetically similar enough to

be considered the same species but separate genomovars. Therefore, the name *Pasteurella atlantica* genomovar *salmonicida* was suggested for the salmon isolates and *Pasteurella atlantica* genomovar *cyclopteri* for the lumpsucker isolates.

The genetic similarities of genomovar *cyclopteri* and genomovar *salmonicida* raises the question if infected lumpsuckers can infect naïve salmon. In 2020 Sandlund et al. (Sandlund *et al.*, 2021) tested this by challenging lumpsuckers and salmon with both genomovars, either by bath or by cohabitation with the challenged lumpsuckers. They found no clinical sign of pasteurellosis in the salmon, but found the lumpsuckers to be equally susceptible to both strains of *Pasteurella* sp. This indicates that farmed salmon can transmit the disease to lumpsuckers in the same fish farms, but infected lumpsuckers possibly cannot transmit the disease to salmon. Moreover, the NVI has not found genomovar *cyclopteri* when sequencing *Pasteurella* sp. isolates from farmed salmon, thus strengthening this conclusion.

Similar to genomovar *salmonicida*, genomovar *cyclopteri* are small rod shaped bacteria, they are non-motile, gram negative and oxidase positive (Alarcón *et al.*, 2016). They grow on blood agar plates with 2% NaCl and form colonies similar in appearance to genomovar *salmonicida*. Infected lumpfish often shows clinical signs like white spot skin lesions, erosion of the tail fin and jaw, cataract, hemorrhage and redness at the base of the fins (Ellul *et al.*, 2019a). There are currently no commercially available vaccines against pasteurellosis in lumpsuckers.

## 1.3 – The immune system and vaccines

The immune system of fish comprise both the innate and the adaptive branches of the immune system (Bone and Moore, 2008; Murphy and Weaver, 2017). The innate immune system recognizes pathogens in generic ways and does not become more effective when re-exposed to the same pathogens. The adaptive immune system recognizes pathogens by specific antigen molecules and provides specific immunity and memory against pathogens with similar molecules for future infections. B-cells and T-cells are both lymphocytes and are responsible for adaptive immunity. T-cells are responsible for adaptive cellular immunity, while B-cells are responsible for adaptive humoral immunity. CD8+ T-cells have T-cell receptors (TcR) which recognize pathogen derived peptides presented on MCH class I and upon binding and activation kill the pathogen infected cells. CD4+ T-cells have TcR which recognize pathogen derived peptides presented om MHC class II and upon activation will differentiate into different subsets of T-helper cells. B-cells have membrane-bound immunoglobulins (Ig) known as B-cell receptors (BcR) and are responsible for production of specific antibodies against the antigen recognized by the BcR. In contrast to mammalian B-cells, teleost B-cells also have phagocytotic abilities, a trait from the innate immune system (Li *et al.*, 2006).

Helper T-cells (CD4+) do not kill pathogens directly, but activate and regulate many immune responses (Bone and Moore, 2008; Murphy and Weaver, 2017). They are important for differentiation of B-cells to plasma cells (effector B-cells), so that the plasma cells can produce antibodies. They are also responsible for activation of effector T-cells (CD8+) and many cells of the innate immune system. Helper T-cells are divided into subgroups like Th1 and Th2. Th1 cells are more important in activation of cellular responses like effector T-cells and macrophages, while Th2 cells are important to activate B-cells and other humoral responses.

Plasma cells are responsible for antibody production, and differentiate from B-cells named so as they, in mammals, mature in the bone marrow (Murphy and Weaver, 2017). Fish lack bone marrow, and their B-cells mature in the head kidney (Bone and Moore, 2008). Plasma cells produce antibodies with the similar specificity as their BcR, and memory B-cells are long lived B-cells that will differentiate into plasma cells if it encounters similar antigens in future infections.

B-cells can be activated either in a T-cell dependent or T-cell independent manner (Murphy and Weaver, 2017). For T-cell dependent activation the BcR of the B-cell will bind the antigen. The antigen is internalized and degraded, and peptides of the antigen are displayed on MHC class II molecules on the surface of the B-cell. A helper T-cell (CD4+) recognizes the peptide with its TcR and binds to the B-cell's CD40 receptor with its CD40L receptor. The Th2 cell will secrete cytokines, and the B-cell will become activated and start clonal expansion. For T-cell independent activation the antigen is often a polymer capable of activating several of the B-cells BcRs and innate membrane receptors simultaneously, and thus initiating clonal expansion without signals from a helper T-cell.

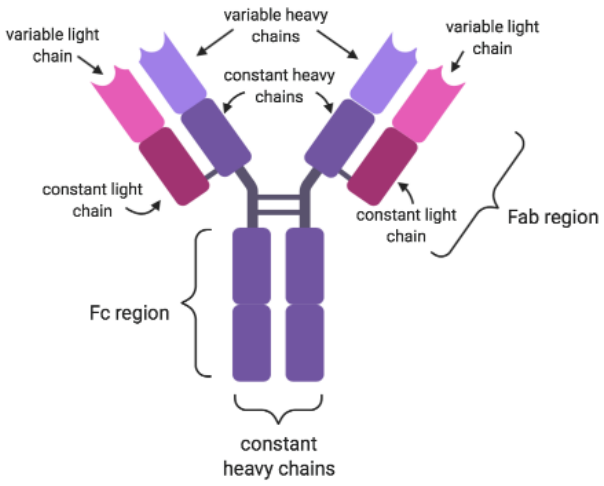


Figure 1 – Structure of a monomer immunoglobulin. The heavy chains are colored light and dark purple, whilst the light chains are dark and light pink. Variable regions of heavy and light chains are colored light purple and light pink. Photo adapted from Rey (2020).

Antibodies, also known as immunoglobulins, are small glycoproteins formed by plasma cells (Bone and Moore, 2008; Murphy and Weaver, 2017). The immunoglobulins are roughly “Y”-shaped and consist of 4 protein chains: two identical heavy chains and two identical light chains (Figure 1). Variation in the amino acid sequence of the heavy chains constant region is responsible for variation in immunoglobulin isotypes like IgM, IgD and IgT which can be found in many teleost fish. In mammals, the plasma cells can switch isotypes whilst still producing antibodies with the same specificity. This is known as isotype switching; however, fish lack this ability. The “upper” part of the Y-shape is the Fragment antigen-binding (Fab) region, and the “lower” part is the Fragment crystallizable (Fc) region. The heavy chain stretches from the FC region to the Fab region. The light chain is only located in the FAB region. The part of the antibody that binds to antigen is named the paratope, and it binds to a region of the antigen known as the epitope. The Y-shaped immunoglobulins can be bound as dimeric, tetrameric, pentameric when they are secreted as antibodies (Figure 2). For example, secreted IgM in bony fish has been described as a tetrameric structure while membrane anchored IgM is a monomer (Hordvik, 2015, Mashoof and Criscitiello, 2016).

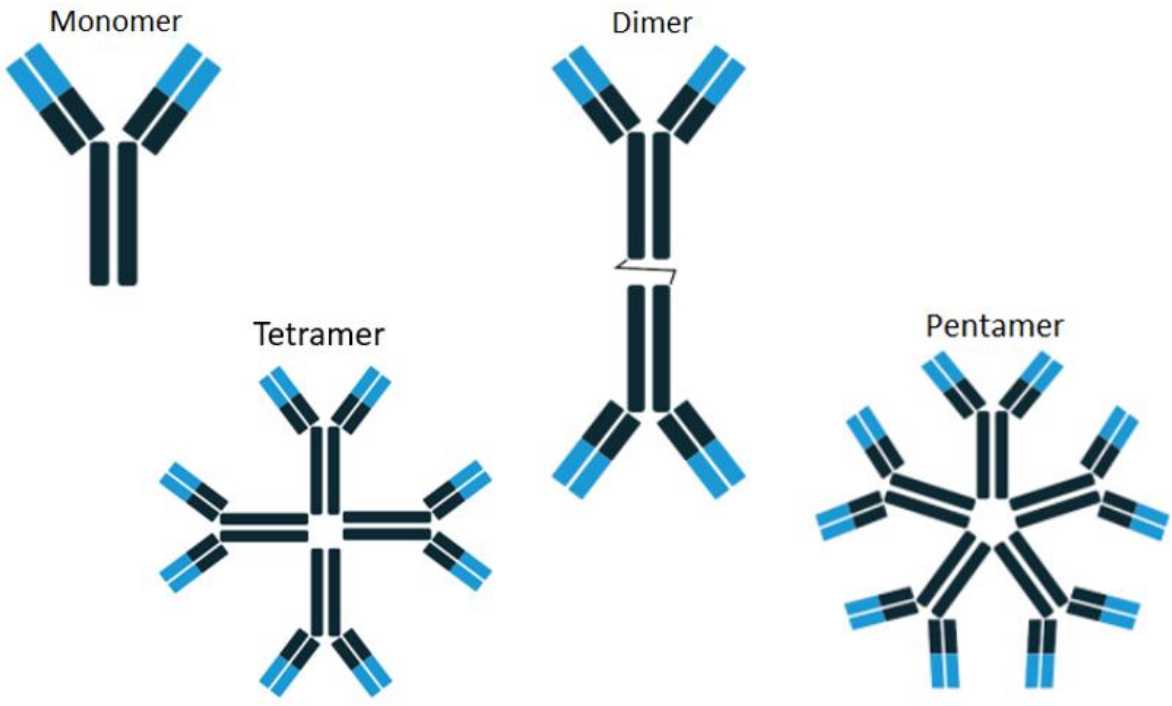


Figure 2 – Schematic overview of antibodies as monomer, dimer, tetramer and pentamer structures. . Photo adapted from Abterra Biosciences (2020).

The antibodies may impact on pathogens in four ways, by neutralization, agglutination, opsonization and activation of the complement system (Figure 3). When antibodies bind to a pathogen's surface, they can block surface proteins and thus neutralizing its ability to bind to host cells and tissues. Also, when antibodies bind to pathogens, the Fc-region of the antibodies can be recognized by phagocytes with their Fc-receptors, and the phagocyte can phagocytize the pathogen, even if the phagocytes lack receptors that identify the pathogen itself. This is called opsonization and is particularly important in defense against bacterial pathogens, while neutralization is considered more important against viral pathogens. Agglutination is when the pathogen is "clumped" together by antibody binding to several antigens simultaneously. This drastically reduces the pathogens mobility and infection ability and increases the chances of it being phagocytized. Antibodies bound to pathogens can also activate the classical pathway of the complement system: a system of over 30 humoral blood borne proteins that can kill pathogens in a cascade reaction when activated.

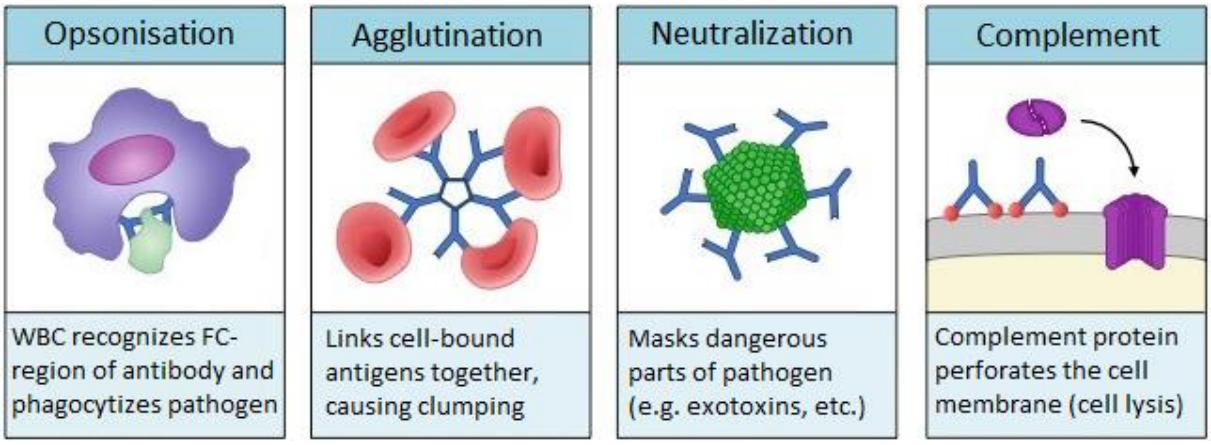


Figure 3 – Illustration of opsonization, agglutination, neutralization, and activation of the complement system by antibodies. Photo adapted from Soegiarto (2021).

Even though teleosts have a well-developed adaptive immune system, the innate immune system is still far from obsolete (Bone and Moore, 2008). The innate immune system is the fastest to respond to pathogens that the fish have had no prior exposure to, which is key to buy time for sufficient adaptive immune response. Additionally, for many infections, the innate immune system fights off the pathogens so fast that there is close to no adaptive immune response. However, both systems are important and must be activated to fight diseases, and both systems are activated through vaccination (Sommerset *et al.*, 2005).

Vaccines work by exposing the immune system to a weakened or dead pathogen, or parts of a pathogen, so that the B-cells and T-cells can create memory cells that lets the adaptive immune system "remember" the antigens of the pathogen for future infections (Bone and Moore, 2008; Murphy and Weaver, 2017). If the fish is infected by the same or similar pathogen to the one presented in the vaccine, its antigen may activate the memory cells, and initiate clonal expansion of both B- and T-cells. The memory B-cell will become plasma cells and more memory B-cells, and the T-cell will become cytotoxic T-cells and more memory T-cells. Most vaccines used in Norwegian aquaculture are composed of pathogens killed with formaldehyde and are mixed with an oil based adjuvant (Sommerset *et al.*, 2005; Mondal and Thomas, 2022). The vaccine is usually injected in the fish's abdomen. It is also possible to use live but weakened pathogens, so that there is a real infection, but no severe disease. Another approach is to use subunits (parts of the pathogen) in the vaccine. It is also possible to use a nucleic acid vaccine, for fish often containing plasmids coding for antigens of the given pathogen. When these plasmids are injected into the fish muscle, production of the antigen will be initiated in the fish muscle cells, and the B- and T-cells will be exposed to the antigen (Sommerset *et al.*, 2005; Brudeseth *et al.*, 2013).

Outer membrane proteins (OMPs) are proteins expressed on the pathogens' surface, and some of them are key for a pathogen to infect its host (Campbell *et al.*, 2017). The immunogenicity of these antigens varies strongly, as bacteria have several mechanisms to prevent a strong immune response from the host (Wizemann, Adamou and Langermann, 1999; Mahanty, Prigent and Garraud, 2015). One mechanism is to hide antigens inside a bacterial capsule, and thus not exposing it to the immune system more than necessary (Merino and Tomás, 2015). Another mechanism is to have plenty of highly immunogenic surface proteins that is not vital for infection, and thus preventing specific immunity to those surface proteins that are necessary for infection. Other mechanisms are to produce immune suppressing cytokines and to avoid host sites with a strong immune presence.

When developing a vaccine, it is useful to target highly immunogenic antigens that are also key for an infection to be successful (Mahanty, Prigent and Garraud, 2015). An example of this is adhesins, as blocking of antigens like these may prevent infection and subsequent disease (Merino and Tomás, 2015). The more immunogenic an antigen is, the less of it is needed in the vaccine, and antibodies with strong affinity to the epitope are often produced, as well as high numbers of memory cells. However, it becomes more difficult to make efficient vaccines if the major infection antigens of the pathogen have low immunogenicity, or if other non-vital antigens are more immunogenic. All of this can explain how a vaccine can succeed in inducing an immune response in the host upon infection but fail to provide protection against the pathogen. However, the use of subunit vaccines can guide protection against specific selected antigens, and thus provide stronger protection against some pathogens.

Formaldehyde is an extremely reactive chemical, and can change surface protein structure, which may reduce the effect of a vaccine based on formaldehyde inactivated pathogens, as the change in surface protein structure may cause specific immunity against a slightly changed epitope (Saito *et al.*, 2005). However, a protein's stability varies greatly, and surface proteins are generally quite stable, so many vaccines based on formalin inactivated pathogens have provided sufficient immunity in the Norwegian aquaculture industry (Sommerset *et al.*, 2005).

The paratope of an antibody can bind with high affinity to an epitope but can usually also bind with a somewhat lower affinity to similar epitopes (Murphy and Weaver, 2017). This is known as cross-reactivity (Figure 4). Cross-reactivity is the reason why a vaccine can still be effective against pathogen surface proteins, even if the proteins have been changed by formaldehyde inactivation. It is also the reason why vaccines can still be effective against mutated pathogens, or pathogens closely related to the target pathogen in the vaccine. An example from the aquaculture industry is the formaldehyde inactivated vaccine against typical furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*), which also give immunity against many atypical strains of *Aeromonas salmonicida* (Gudmundsdóttir and Björnsdóttir, 2007).

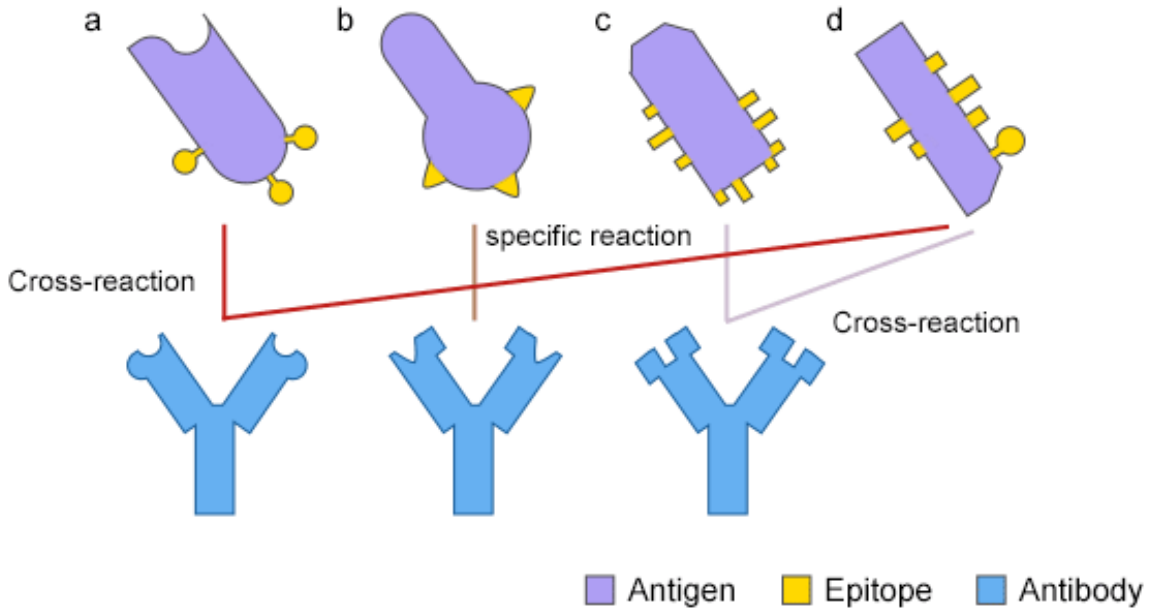


Figure 4 – Illustration of cross-reaction and specific reaction between antibodies' paratopes and antigens' epitopes. Photo adapted from Cusabio Technology LLC (2021).

The vaccines can be distributed by abdominal or muscular injection, by mixing the vaccine in the fish's water (immersion vaccine), or by mixing it in the fish's feed (oral vaccine) (Sommerset *et al.*, 2005; Brudeseth *et al.*, 2013). Oral vaccines are the least stressful for the fish, however the obtained protection is often poor. Vaccination by immersion is less stressful than vaccination by injection, but for many pathogens it is difficult to make an efficient immersion vaccine that provide long lasting protection. Oil based injectable vaccines are usually the most efficient of these three types. There are multiple ways of measuring the effects of a vaccine, but the most commonly and regulatory approved method is by calculating the relative percentage survival (RPS), meaning how many percent more survival is obtained in the vaccinated group compared to an unvaccinated group during an experimental infection challenge.

After vaccination, the immune system needs time to test random BcR of B-cells against the epitopes of the antigens (Murphy and Weaver, 2017). Thus, it takes some time for the production of specific antibodies to begin, but as the right type of antibodies are made, the levels of antibodies in the fish sera rises quickly. This is known as the primary response. If the vaccinated animal is re-vaccinated or infected with the targeted pathogen, then the immune system will have memory B-cells ready to induce the production of specific antibodies, much faster than that of the primary response. This is known as the secondary response and will induce the production and storage of even more memory B-cells. A third vaccination and/or infection may induce even more antibody production and memory B-cell production and storage.

## 1.4 – Vaccines and antibiotics in Norwegian aquaculture

In 1987 about 50 metric tons of antibiotics were used in Norwegian aquaculture whilst only producing 50.000 metric tons of fish (Sommerset *et al.*, 2005; Brudeseth *et al.*, 2013). Only two years later antibiotic usage dropped to about 20 metric tons, while the production volume of fish had almost doubled. In the year 2020, the industry produced over 1.4 million metric tons of salmonids, whilst only using 0.229 metric tons on antibiotics (Directorate of fisheries, 2021; Litleskare, 2022). This drastic reduction in antibiotic usage is mainly due to use of commercially available vaccines in the Norwegian aquaculture industry.

In the 1980s the industry probably would have crashed due to bacterial infections, if it had not been for antibiotics against bacterial pathogens such as *Vibrio* spp. (Sommerset *et al.*, 2005). As use of antibiotics can lead to antibiotic resistant bacteria, this solution was only considered to be temporary, and a better solution was needed (Campbell *et al.*, 2017). In the USA in the 1970s fish immersion vaccines against *Vibrio* spp. based on formaldehyde inactivated bacteria was proven effective, and

similar vaccines was used in Norwegian aquaculture in the 1980s which successfully reduced the antibiotic usage. Later, around 1990, the usage of antibiotic was on the rise again, due to an increase in infections by *Aeromonas salmonicida* subspecies *salmonicida*. Immersion and water-based injection vaccines did not provide sufficient protection against the disease (furunculosis), thus injection vaccines containing formaldehyde inactivated bacteria formulated in an oil-based adjuvant, were developed and proven effective. This type of oil-based injection vaccine containing inactivated pathogens has been the most common vaccine used in Norwegian aquaculture since. Today, multicomponent vaccines, meaning they have multiple different pathogens in the same vaccine, are commonly used as they provide protection against multiple diseases without needing to handle the fish multiple times. In 1995 recombinant vaccines were introduced to Norwegian aquaculture with Norvax®Protect-IPN against the IPN virus (Frost and Ness, 1997). In 2017 Elanco gained marketing authorization for a nucleic acid-based vaccine against pancreas disease (PD), caused by salmonid alphavirus (SAV) in Norwegian Atlantic salmon, and the vaccine was shown to give significantly better protection against the virus than the commercially available oil based SPDV vaccine ALPHA JECT Micro 1 PD in a study by Thorarinsson *et al.* (2021).

For pathogen induced diseases with no commercially available vaccines, it is possible to use autogenous vaccines (Ramírez-Paredes *et al.*, 2019; Vaxxinova, 2021). Autogenous vaccines are tailor-made based on a specific pathogen harvested from the fish farm where the vaccine will be used. Autogenous vaccines are not to be used if other vaccines are commercially available. Autogenous vaccines do not need market authorization for sales, and thus do not need to fulfill all requirements to document its efficiency (Legemiddelforskriften, 2009).

There are currently no commercially available vaccines against pasteurellosis in Atlantic salmon, and antibiotics such as oxytetracycline, sarafloxacin and amoxicillin seem to have little to no effect on the course of the disease, even though the bacteria is sensitive to multiple antibiotics used in aquaculture when tested in vitro plate diffusion (Jones and Cox, 1999; Valheim *et al.*, 2000; Legård and Strøm, 2020; Sandlund *et al.*, 2021). More research is needed to develop a commercially available vaccine for this disease. A challenge model needs to be established for documentation of RPS after vaccination, and importantly an optimal bacterial candidate of *Pasteurella* sp. must be identified for vaccine incorporation. The latter is what this thesis aims to provide more knowledge on.

Members of the family *Pasteurellaceae* may have been found intracellularly in phagocytic immune cells of Atlantic salmon, and this is also suspected to be the case in lumpsucker infected with *P. atlantica* genomovar *cyclopteri* (Jones and Cox, 1999; Ellul *et al.*, 2019b; Legård and Strøm, 2020). This may explain why antibiotic treatments do not cure the disease, as the bacteria can be protected from

the antibiotics inside phagocytic cells, which can result in reoccurrence of disease shortly after ended antibiotic treatment. This could also protect the bacteria from the effect of antibodies targeting the bacterium, which may make vaccine development a more challenging task. Therefore, it may be important to make vaccines aimed at antigens that are key to early stages of the bacterial infection. Ellul et al. (2019a) tested a vaccine against pasteurellosis in lumpsucker, which gave a sufficient specific antibody response, but insufficient protection against the disease. This could indicate that the bacteria are facultative intracellular, but another explanation could be that the formaldehyde inactivation have changed the target proteins epitope too much for it to give sufficient protection against the live bacteria.

## 1.5 – Aims of the study

The number of outbreaks of pasteurellosis in Norwegian salmon farming has increased rapidly since 2018, and more knowledge is needed in order to make efficient commercially available vaccines (Sommerset *et al.*, 2021). Little is known about *P. atlantica* genomovar *salmonicida's* surface proteins, immunogenic components, and virulence factors, and it is also not known if this is a homogenous group or if there are differences between isolates of this group when it comes to expression of surface proteins after in vitro culturing. By vaccinating Atlantic salmon with three isolates of *P. atlantica* genomovar *salmonicida*, harvested at different farm sites, we produced antisera targeting the different isolates. Further, the specificity of these antisera was studied to increase our knowledge of the immunogenic components of these isolates, the cross reactivity of the antibodies produced as well as the salmon's immune response towards these isolates. Specifically, the aims of the study were to:

- Produce three oil-based vaccines and to vaccinate Atlantic salmon to produce antisera against three isolates of *P. atlantica* genomovar *salmonicida*.
- Examine the cross-reactive properties of the antibodies produced targeting the three isolates of *P. atlantica* genomovar *salmonicida*.
- Examine cross-reactivity of antisera targeting genomovar *salmonicida* on antigens of genomovar *cyclopteri*.
- Examine immunogenic components of these isolates.
- Examine adaptive immune response against these isolates.

# 2 – Materials and methods

## 2.1 Bacteria

Four isolates of *P. atlantica* genomovar *salmonicida,* harvested from Atlantic salmon diagnosed with pasteurellosis, were included in this study. As well as one isolate of *P. atlantica* genomovar *cyclopteri*, harvested from lumpsuckers diagnosed with pasteurellosis. Ellul *et al.* (2021) have used the lumpsucker isolate in similar experiments with pasteurellosis in lumpsuckers, and referred to the isolate as UiBP1-2013, while it will be referred to as RK-1 in this thesis. The salmon isolates originate from fish diagnosed with pasteurellosis from commercial salmon farms in Vestland county, Norway (Table 1). The isolates will be referred to as PaL-1, PaL-2, PaL-3, PaL-4 and RK-1 in this thesis. The PaL-4 isolate was isolated as a part of this master thesis.

Table 1 – Overview of *P. atlantica* isolates included in the thesis, what fish species they were isolated from, which location they were isolated from, and in which year they were isolated.

| Isolate name | Host species | Location | Year |
|---|---|---|---|
| PaL-1 | Atlantic salmon (S*almo salar* L.) | Vestland county (Norway) | 2019 |
| PaL-2 | Atlantic salmon (S*almo salar* L.) | Vestland county (Norway) | 2020 |
| PaL-3 | Atlantic salmon (S*almo salar* L.) | Vestland county (Norway) | 2020 |
| PaL-4 | Atlantic salmon (S*almo salar* L.) | Vestland county (Norway) | 2021 |
| RK-1 | Lumpsucker (*Cyclopterus lumpus* L.) | Vestland county (Norway) | 2013 |

### 2.1.1 Culturing bacteria

The bacterial isolates were cultured on either 2% NaCl sheep blood agar (BA) plates (NVI) or in Tryptic Soy Broth (TSB) (BD, Lot# 1068445) supplemented with 0.5 % NaCl and 10% Foetal Calf Sera (FCS) (Gibco – Lot# 2094466RP), hereafter called TSB+. Cultivation of bacteria in TSB+ was performed using 50 ml centrifuge tubes (SARSTEDT AG & Co). The bacteria, from freeze stock cultures (1 ml), were suspended in TSB+ (44 ml) and incubated in a shaking incubator (Infors AG, model Minitron) at 200 RPM and 20 °C for 24hours. For the BA plate cultivation, the bacterial samples were smeared on the plates using an inoculation loop, and the plates incubated in an incubator (Sanyo™, MIR-154PE) at 15 °C for a minimum of 24 hours.

### 2.1.2 Preparations of glycerol stocks of PaL-1, PaL-2 and PaL-3

These three isolates were obtained from the Fish immunology group at the University of Bergen, as frozen 1 ml bacterial culture stocks. The cultures were first thawed, then transferred to 44 ml TSB+ (Appendix) and incubated in a shaking incubator (Infors AG, model Minitron) at 200 RPM and 20 °C for 24 hours. Then 4.4 ml of ≥99% glycerol (Sigma-Aldrich) was pipetted to each of the three tubes. Lastly, 1 ml of the solutions was added to 2.0 ml cryo tubes (VWR), and frozen at -80 °C (Thermo Scientific™, Revco™) to provide stocks for subsequent analysis.

## 2.2 Isolation and identification of PaL-4

The PaL-4 isolate originated from diseased salmon from a fish farm in Vestland county, Norway. Three individual 4 kg sized fish were examined externally and internally for clinical signs of disease. Bacterial samples were taken by aseptically injecting inoculating loops into the fish head kidney, spleen, and heart ventricle, and smearing the sample on BA plates. The head kidney and heart ventricle were cut open using a sterile scalpel before sampling using the inoculation loop. The BA plates were incubated at 15 °C for 24-48 hours before single bacterial colonies were transferred and smeared onto new BA plates. This was repeated once more, to tertiary BA plates, to ensure pure cultures.

Parts of one colony were streaked onto BA and incubated at 15 °C for storage until identification was confirmed. The rest of the colony was dispersed in a 1.5 ml Eppendorf tube (Corning incorporated) filled with 50 µl nuclease free water (Sigma-Aldrich), and heated at 98˚C for 10 min before it was centrifuged at 1300 x g for 2 min. The supernatant was harvested into sterile Eppendorf tubes and used as template for 16S rRNA sequencing.

Before amplification by polymerase chain reaction (PCR) the supernatant was diluted 10 folds by pipetting 10 µl of the supernatant to new 1.5 ml microtube (Corning incorporated), and then adding another 90 µl of nuclease free water (Sigma-Aldrich) to the tubes to make different dilutions of the supernatant.

The 16S rRNA gene was amplified using universal primers 27F: 5’-AGAGTTTGATCMTGGCTCAG-3’ and 1525R: 5’-AAGGAGGTGWTCCARCC-3’ (Collins *et al.*, 1991). The reagents (Table 2) were mixed in 0.2 ml PCR tubes, and the tubes were then transferred to a thermal cycler (Applied Biosystems, 2720 Thermal Cycler) for the PCR-reaction. Following the settings in Table 3.

Table 2 – 16S rRNA gene amplification reagents.

| Reagents (Thermo Scientific) | Volume (µl) |
|---|---|
| Nuclease free water | 31.5 |
| 5x Phusion buffer | 10 |
| 10mM dNTP | 1 |
| 16S 27 forward primer | 2.5 |
| 16S 1525 reverse primer | 2.5 |
| Phusion polymerase | 0.5 |
| Sample supernatant | 2 |

Table 3 – Thermal cycler program for PCR assay.

| | Temp (°C) | Time (min) | Number of cycles |
|---|---|---|---|
| First Denaturing | 98 | 3 min | 1 |
| Denaturing | 98 | 30 sec | 30 |
| Annealing | 58 | 30 sec | |
| Extension | 72 | 1 min | |
| Final Extension | 72 | 10 min | 1 |
| End stage | 4 | o/n | |

The PCR products were visualized on an 1% agarose gel to confirm a successful PCR reaction. The samples were traced by mixing 5 µl of the PCR product mixed with 2 µl 5x loading buffer (BlueJuice™ Gel loading Buffer, Thermo Fisher Scientific), before 7 µl of mix was pipetted into each gel chamber. The gel was run at 80V for 45 minutes in 1xTAE buffer (Appendix) before visualizing the bands using UV light (Syngene, G:Box).

### 2.2.1 PCR product purification and 16S rRNA sanger sequencing

The PCR product from 2.2.3 was purified using a GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) following producers' descriptions. Then the PCR product was quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies Inc., model NanoDrop™ 1000 Spectrophotometer). The purified PCR product

was prepared for sanger sequencing by mixing with Big-Dye, Sequencing buffer, water and forward or reverse primer before running the PCR reaction (Table 4). Sequencing was performed by the DNA sequencing facility at the High-Technology Centre of Bergen, Norway. The results were analyzed with BLASTN (Zhang *et al.*, 2000; Morgulis *et al.*, 2008) to confirm if the isolated bacteria was *P. atlantica*.

Table 4 – Sanger sequencing reaction reagents.

| Reagent | Volume |
|---|---|
| Big-Dye version 3.1 | 1 µl |
| Sequencing buffer | 1 µl |
| Template | 200 ng |
| Primer (forward or reverse) | 3.2 pmol |
| Milli-Q (Merck Millipore, model Milli-Q® Advantage A10® System) | 10 µl total volume |

## 2.3 – Fish and rearing conditions

The vaccination was performed at the Industrial and Aquatic Laboratory (ILAB) in Bergen. The Atlantic salmon was acquired from ILAB at 127 g (±14 SD) and 21 cm (±1 SD) average at the beginning of the experiment. The fish had no signs of infections or mortality and had been acclimatized to the rearing conditions for two weeks before onset of the experiment. The fish was reared at 12 °C and 34‰ salinity. The photoperiod was set to 12L:12D, and feeding was done by automatic feeders and according to appetite during light hours. The fish was starved for 48 hours before vaccination and blood collection. The feed was 3mm Nutra Olympic by Skretting AS and was upped to 4mm Nutra Olympic once the average weight of the fishes passed 160 g. The water flow was set to 1500 L/min, and oxygen saturation was set between 90%-95% throughout the experiment. The rearing tanks had a volume of 1000 L, shaped as a cube with rounded edges, and had no recirculation of the water. Oxygen saturation and salinity was monitored daily, and the fish health and behavior were checked every day throughout the experiment.

## 2.4 – Preparation of vaccines and vaccination

Atlantic salmon were vaccinated against three isolates of *P. atlantica* genomovar *salmonicida*: PaL-1, PaL-2, and PaL-3.

The bacteria for vaccine incorporation were cultured in 44 ml TSB+ (Appendix) in a shaking incubator (Infors AG, model Minitron) at 200 RPM and 20 °C for 24 hours before measured for cell count using a Microbial Cell Counter (Roche Innovatis AG, CASY® Model TT 150 µm). About 25 ml of the bacterial cultures were pipetted to each of 50 ml centrifuge tubes (SARSTEDT AG & Co.), on a weight scale (Thermo Fisher Scientific Inc, Model PPS2102). Then a volume, equal to 1% of the volume of bacterial isolate cultures, of 37% formaldehyde solution (Sigma-Aldrich) was pipetted to each of the 50 ml centrifuge tubes. The tubes were placed in a shaking incubator (Infors AG, model Minitron) at 200 RPM and 20 °C for 15 minutes, before the content of the tubes were transferred to new sterile 50 ml centrifuge tubes and replaced in the incubator for another 75 minutes. Lastly, 100 µl of the contents was smeared on to BA plates as control of inactivation, and 100 µl of non-inactivated bacterial cultures smeared on BA plates serving as a positive control. The inactivated *Pasteurella* isolates were stored at 4°C, while the BA plates were placed in an incubator (Sanyo™, model MIR-154PE) at 15 °C for three days before they were controlled for growth.

Ten fish were vaccinated with each bacterin, in addition 10 fish served as non-vaccinated controls and were injected with sterile PBS (Lonza Group AG, Lot# 0000997114). For each fish group, injected with the different bacterins, the fish were vaccinated three times. For the first injection, the vaccine was diluted 1:1 with Freund's incomplete adjuvant (Sigma Corporation, lot# SLBN5308V), the second vaccine contained only bacterins, and the third vaccine contained bacterins diluted 1:1 in PBS (Table 5). The non-vaccinated control group was injected with sterile PBS and kept in a separate but similar fish tank than the vaccinated groups. The vaccinated groups receiving the *P. atlantica* genomovar *salmonicida* bacterins were kept in the same fish tank but were tagged using Visible Implant Elastomere (VIE) (Northwest Marine Technology) set subcutaneously over the left eye. The VIE tag color differed between the three vaccine groups. Four weeks passed between vaccination numbers 1 and 2, and two weeks passed between 2 and 3. 12 days after the third vaccination, the fish were euthanized using a Finquel (MSD Animal Health, Finquel) overdose, and blood were sampled.

Table 5 – Overview of date, added adjuvant, volume of injection and dilution of inactivated bacteria during vaccination and blood sampling events.

| Event | Date | Adjuvant added (+/-) | injection volume (µl) | Dilution of bacterins |
|---|---|---|---|---|
| Vaccination number 1 | 10.09.2021 | + | 100 | 1:1 |
| Vaccination number 2 | 08.10.2021 | - | 100 | No dilution |
| Vaccination number 3 | 22.10.2021 | - | 100 | 1:1 |
| Blood sampling | 03.11.2021 | Not relevant | Not relevant | Not relevant |

The first injection dose was prepared by pipetting 600 µl of inactivated bacteria to each of two 2.0 ml tubes (VWR International) per isolate, and then supplying 600 µl of Freund's incomplete adjuvant (Sigma Corporation, lot# SLBN5308V) to the tubes. Thus making 2, 4 ml of mixture per isolate. The tubes were placed in a homogenizer (MP Biomedicals, FastPrep-24™ 5G) and vortexed at 4 m/s for 10 seconds to homogenize the inactivated bacterial culture with the adjuvant. Then two 1 ml plastic syringes per isolate was filled with the vaccine, a 0.5 mm diameter needle (HSW) was put on the syringe, and the syringes were emptied for air. The second and third injection dose used the same batch of inactivated bacteria but did not include adjuvant. The third injection dose was diluted 1:1 with PBS.

The fish were starved for 48 hours before vaccination and blood collection assays. In the rearing facility at ILAB, a 250L water holding tank was filled with about 100L of water. An aeration stone was placed in the holding tank, providing atmospheric air and sufficient oxygen levels. And a 15L anesthetics bucket was provided by mixing 10L of water with 1 gram of Finquel (MSD Animal Health, Finquel).

The first vaccination started by gently transferring all the 41 fish from their 1000L rearing tanks to the 250L holding tank using a fine meshed hand net. Then four fish at a time were transferred from the holding tank, and into the anesthetics bucket. About 45 seconds after fishes lost equilibrium, the fish was taken out of the anesthetics bucket, weighed, and measured for length, VIE-marked (Northwest Marine Technology), and vaccinated by intraperitoneal injection in mid abdomen. However, the first ten fish were not VIE-marked and were injected with sterile PBS (Lonza Group AG, Lot# 0000997114), and thus functioned as non-vaccinated controls. Contrary to the vaccinated fish, the non-vaccinated control fish were not transferred back to the same fish tank they came from but were transferred to a

new separate fish tank. Fish 11-20 got the PaL-2 vaccine, fish 21-30 got the PaL-1 vaccine, and fish 31-41 got the PaL-3 vaccine.

The next two injections were performed in a similar manner, but with a few key differences. The first ten negative control fish were not injected more than the first time, and thus was only handled once before blood sampling. Also, the vaccinated fish were only VIE-marked during the first injection, as the mark was visible throughout the trial. During the two last injections, and the blood collection, all vaccinated fish were measured for weight and length. As scale loss was observed after the first and second injection, 1 ml of 540 mg/ml of sedation Isoeugenol (AQUI-S New Zealand Ltd, AQUI-S®) was added to the fish rearing tank for the third vaccination and blood collection, before transferring the fish to the holding tank. The fish were transferred to the holding tanks after a maximum of 3 minutes of sedation.

### 2.4.1 – Blood collection and extraction of immune sera

Preparation for the blood collection assay was done quite similarly as for the vaccination assay. However, the anesthetics concentration was doubled in the anesthetics bucket, and the fish were killed by anesthetics overdose. Anesthetic overdose was achieved about 45 seconds after the fish's gill frequency reached zero. Weight and length were measured in the same manner as during vaccination. Following, the blood was extracted by using a 1 ml syringe with a 0.5 mm diameter needle (HSW) injected in the caudal vein posterior of the fish anal fin. For each fish, the syringe was filled about 2-5 times, and the blood was transferred from the syringe into 5ml blood sample collection tubes (BD). The blood sample collection tubes were stored at 4˚C for 24 hours to allow the blood to coagulate. Lastly most of the fish's abdomens were opened to examine for side effect from the vaccine, like adherence and melanin spots. The immunization trial was applied for to the Norwegian Food Safety Authorities and designated the approval identification Id: 28217.

24 hours after the blood collection, the blood sample collection tubes were placed in a centrifuge (Beckman Coulter Inc., Allegra X-15R Centrifuge) at 393 xg for 10 minutes at 4 °C. Then the sera were pipetted and allocated to 1.5 ml microtubes (Corning incorporated,) and stored at -20 °C.

## 2.5 – Serology

Enzyme Linked Immuno Sorbent Assay (ELISA) was performed to measure the levels of specific antibodies in sera from the vaccinated fish. Western blot (WB) analysis was performed to analyze binding of antibodies in the immunosera to proteins of specific sizes.

### 2.5.1 – Preparation of antigens for ELISA and protein gels.

Freeze-dried bacteria of the isolates PaL-1, PaL-2, PaL-3, and RK-1 were used as antigens. Bacterial isolates were cultured as described in section 2.1.1 and harvested after 24h in late exponential growth phase. The bacterial suspensions were centrifuged (Beckman Coulter Inc., Allegra X-15R Centrifuge) at 2500 xg for 15 minutes at 4 °C, and the supernatant was gently removed by using a pipette. Then 500 µl of Milli-Q (Merck Millipore, model Milli-Q® Advantage A10® System) was added to each tube, and the pelleted bacteria resuspended. The solutions were transferred to sterile pre-weighted 50 ml centrifuge tubes (SARSTEDT AG & Co), and stored at -80°C.

At the day of the freeze-drying, holes at about 2 mm in diameter was pierced with a needle in the screw caps of the 50 ml centrifuge tubes, and the tubes were placed in the freeze-drying machine (Martin Christ Gefriertrocknungsanlagen GmbH, Alpha 1-2 LDplus). For the next 24 hours, the machine created sufficient vacuum and heat to freeze-dry the solutions to a powder of freeze-dried bacteria.

To tear open the cell walls and exposing more antigens for the assays, the freeze-dried bacteria were sonicated before use as antigens. Firstly, the centrifuge tubes containing the freeze-dried bacteria were weighted without the screw cap and the weight difference between this measurement and post freeze-drying measurement, was used to calculate the weight of the freeze-dried bacteria. Then 1 ml of EDTA PBS buffer (Appendix) was pipetted per 10 mg of freeze-dried bacteria to each of the tubes. The contents in the tubes were sonicated in a sonication machine (Sonics & Material inc., model Vibra-Cell VCX130) with a stepped microtip diameter of 3 mm (Sonics & Material inc., part 630-0422), for 2 minutes each on ice. Then 2.5 ml of the solution in each tube were pipetted to each new 50 ml tube, and 22.5 ml of EDTA PBS solution (Appendix) was pipetted to each of the tubes, to make a final concentration of 1 mg/ml of sonicated bacteria. Lastly, the solutions were aliquoted to 1.5 ml microtubes (Corning incorporated) to provide stock solutions for the following analysis. The microtubes were stored at -20°C.

### 2.5.2 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was performed using the stock solutions described in section 2.5.1 as antigen, and the sera described in section 2.4.1 as test salmon immune sera. As well as one immune sera from lumpsuckers vaccinated with a similar vaccine based on the RK-1 isolate, provided by the Fish immunology group at the University of Bergen.

First, titration curves (ten-fold dilutions) were compiled for all antisera to identify the optimal dilution of antisera for further analysis, and to compare against sera from non-vaccinated fish. Further, cross-reactivity of specific antibodies present in the antisera were analyzed across the isolates. See Appendix B for an overview of the assays.

A microtube containing antigens at dilution 1mg/ml was thawed and diluted 1:100 in PBS (Appendix). 100 µl of this solution was supplied to all wells of 96 well plates (Thermo Scientific™, Nunc™). The plates were then stacked on top of each other, with an empty plate on the top, and placed in a sealed plastic bag with wet paper towels in the bottom, to prevent the content of the wells from vaporizing. The bag was stored at 4°C overnight.

The second day of the ELISA each well of the plates were washed three times using PBS-Tween (Appendix) using a microplate washer (BioTek Instruments Inc, model 405LSR). Then 200 µl of PBS blocking solution (Appendix) was pipetted to each well and the plates were incubated in the plastic wet towel bag at room temperature (Rt) for one hour. The plates were then washed three times, and 50 µl of diluted antisera (diluted with PBS-Tween solution) was added to the wells. To wells functioning as negative controls, 50 µl of PBS-Tween solution only was added. The plates were left for incubation in the plastic wet towel bag in an incubator (Sanyo™, MIR-154PE) at 15°C overnight.

The plates were washed three times in the microplate washer (BioTek Instruments Inc, model 405LSR), and 50 µl of diluted (1:2000) rabbit anti-salmon IgM antisera was added to each well of the plates. The plates incubated for 2 hours at Rt in the plastic wet towel bag. Then goat anti-rabbit Ig conjugated with HPR antisera (Dako Denmark A/S) was diluted 1:2000, the plates were washed three times, and 50 µl of this antiserum was added to all wells of the plates. The plates incubated for 1 hour at Rt in the same bag. Then the plates were washed three more times, and 50 µl of peroxidase substrate solution (Appendix) was added to all well of the plates. After exactly 6 minutes, the reaction was stopped by adding 50 µl of 2.5M $H_2SO_4$ solution (Appendix) to the wells. Then the absorbance of the solutions was measured at 492nm in a spectrophotometric microplate reader (Tecan Group Ltd, model SPECTRA Fluor F129003).

## 2.6 SDS-PAGE gels, silver staining and Western blot

Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining were preformed to display the protein profiles of the PaL-1, PaL-2, PaL-3 PaL-4 and RK-1 isolates.

The bacteria were cultured as described in 2.1.1 and centrifuged at 2500 xg and 4˚C for 10 minutes. The supernatant was removed, and the pellet resuspended in 20 ml Milli-Q (Merck Millipore, model Milli-Q® Advantage A10® System). These five solutions, as well as sonicated solutions of PaL-1, PaL-2 and PaL-3 described in section 2.5.1 were used as template for the gels. For preparation they were mixed 1:1 with SDS Reducing Buffer (Appendix) supplied with β-mercaptoethanol (Sigma-Aldrich) (1:20) and heated at 98˚C for 5 minutes in a heat block (Eppendorf, model 5355).

The gels (12% acrylamide Resolving gel and 4% Stacking gel) were prepared according to the method of Laemmli (Laemmli, 1970). A Mini Protean Tetra Cell (Bio-Rad) was used for the electrophoresis. Bacterial samples (10 µl) were supplied to the wells and unstained low range SDS-PAGE standard (Bio-Rad) diluted 1:20 in the SDS Reducing Buffer, treated in the same manner as the bacterial samples, were used as ladder (5 µl). The gels were electrophoresed at 190V for 50 minutes in a vertical electrophoresis chamber (Bio-Rad, Mini-PROTEAN Tetra Cell).

Two of these gels were used for silver staining, whilst four were used for WB. For the silver stain gels, the unstained SDS-PAGE standard (Bio-Rad) was used as ladder, and for the WB a Kaleidoscope™ WB standard (Bio-Rad) was used.

The two gels for silver staining were fixed in fixative solution (Appendix) for 20 minutes, and then washed twice with Milli-Q (Merck Millipore, model Milli-Q® Advantage A10® System) for 10 minutes each time. The gels were stained using the Silver Stain plus kit (Bio-Rad) for about 20 minutes until clear protein band were formed and the reaction was stopped by adding 5% acetic acid solution (Appendix). The gels where photographed (Bio-Rad, Universal Hood II).

The four gels for WB were soaked in blotting buffer (Appendix) for 30 minutes, and then mounted into a sandwich of sponges (Bio-Rad, lot# 181819), paper (Ahlstrom Falun, lot# 2925), and a 0.2 µm pore nitrocellulose membrane (Bio-Rad, lot# G9906121) (Figure 5). The sandwich was placed in an electrophoresis cell (Bio-Rad, Mini-PROTEAN II), and ran for 60 minutes at 100V to transfer the proteins from the gel on to the nitrocellulose membrane. The membrane was washed in TBS (Appendix) for 1 minute in a glass container, and then blocked in TBS blocking solution (Appendix) on a flat orbital shaker (IKA, KS 260 basic) at 50 rpm for 60 minutes. Then the membranes were washed twice in the same manner as all further washing steps of the WB: with TTBS (Appendix) for 5 minutes at 50 rpm on the swirl machine. Then 100 µl of antisera from one PaL-1 vaccinated fish, one Pal-2 fish, one PaL-3

fish, and one control fish were diluted 1:100 in antibody buffer (Appendix) and supplied to separate membranes. The four membranes with sera were incubated on the swirl machine at Rt and 50 rpm overnight.

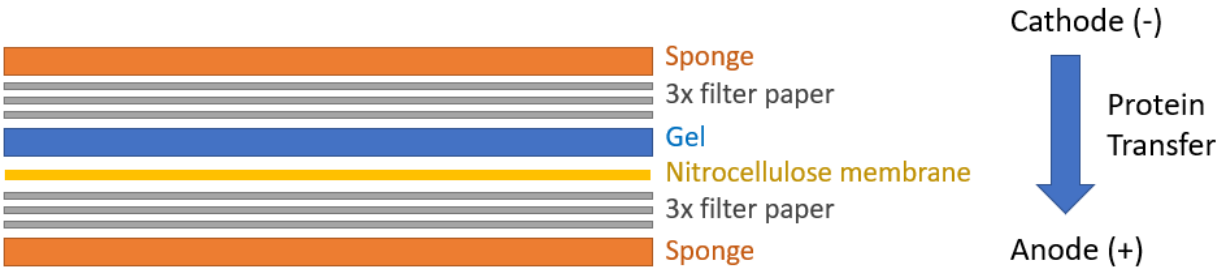


Figure 5 – Western blot protein transfer setup. Proteins will be transferred from cathode towards anode and will thus be transferred from the gel to the nitrocellulose membrane.

The membranes were washed twice, before the rabbit anti-salmon IgM antisera was diluted 1:2000, and 10ml of the solution was added to each membrane for incubation for 120 minutes. The membranes were then washed twice, and then goat anti-rabbit Ig conjugated with HPR antisera (Dako Denmark A/S) diluted 1:1000 was added to the membranes for incubation for 90 minutes. The membranes were washed twice, transferred to new clean glass containers, incubated in TBS for 5 min before they were supplied with 1:1 mixed substrate solution (Bio-Rad, lot# 102030505) and incubated for 5 minutes. The membranes were removed from the box and photographed with an exposure time of 1.1 seconds (Bio-Rad, Universal Hood II).

## 2.7 Gross pathology of salmon diseased with *P. atlantica* genomovar *salmonicida*

Close to the end stages of this master thesis, we got the opportunity to visit a commercial Atlantic salmon farm with elevated mortality due to an outbreak of pasteurellosis. The farm was located in Vestland county, Norway. The freshest diseased fish was examined for clinical signs of disease, and the pathology found was photographed with a mobile phone (Realme, RMX 2111). Bacterial samples were not collected for further research, but head kidney tissue was sampled for qPCR test by an authorized aqua medicine biologist and later confirmed the presence of *P. atlantica*.

## 2.8 Phylogenomic analysis

Six *Pasteurella* isolates were included in phylogenomic analyses to assess the degree of similarity in genes between the isolates. The isolates were: the salmon isolates PaL-1 and PaL 2, the lumpfish genomovar *cyclopteri* isolates RK-1 and NVI 9100 previously described by Ellul et al. (2021) and two *P. skyensis* isolates, DSM24204 (Foster et al., 2000) and CP016180 (Hansen, Bojesen and Planet, 2016). The genomes of the PaL-1 and PaL-2 isolates were prepared by the Fish Immunology group and ACD Pharmaceuticals (not yet published). The genomovar *cyclopteri* isolates corresponds to genomes published in Ellul *et al.* 2021, and the *P. skyensis* genomes are accessed through the GenBank (NCBI). The RK-1 isolate is the same that has been used previously in this thesis.

Genome assembly, annotation and pan-genome analyses were performed by ACD Pharma. The genomes were assembled using spades 3.15.2 (Nurk *et al.*, 2013) and annotated using the Prokka v1.12 pipeline (Seemann, 2014), and the protein coding sequences of all six isolates were compared using a blast all-against-all approach and grouped into orthologous clusters using Roary (Page *et al.*, 2015). Orthologous clusters were curated to exclude significantly divergent singletons, as they are likely to be the result of erroneous assembly or annotation. Furthermore, a pan-genomic presence/absence matrix was constructed, including each gene cluster and each genome. Hierarchal single-linkage clustering analysis of this matrix was performed in R to construct a pangenome heatmap overview using the heatmap (R Core Team, 2020). The packages used were gplots, dendextend and DECIPHER, MASS (Ripley *et al.*, 2002; Galili, 2015; Warnes *et al.*, 2016; Wright, 2016). RAxML-ng and the packages DECIPHER and MASS, were used to construct a Maximum Likelihood (ML) phylogenetic supertree, using core genes included in a multi-locus multiple alignment scheme, and to determine the phylogenetic distances (Kozlov *et al.*, 2019).

## 2.9 Statistics

All data were statistically analyzed using GraphPad Prism Windows version 5.00, Microsoft Excel for Microsoft 365 MSO Version 2112 and R program with the packages tidyverse and readxl (Graphpad, 2007; Wickham and Bryan, 2019; Wickham *et al.*, 2019; R Core Team, 2020; Microsoft Corporation, 2022). GraphPad Prism was used to make vertical scatter plots of the absorbance from the spectrophotometric microplate readings of the ELISA assays, and Microsoft Excel was used to make line charts of the same absorbance. Mann Whitney U Tests was used in R to test for significant differences between vaccine groups and control groups of the ELISA assays, and to test for significant differences in growth during the vaccine trial. Appendix B table 8 and 9 displays all p-values for these calculations. Significant differences were based on p-values and considered significant if $p < 0.05$.

# 3 – Results

## 3.1 – Gross pathology of fish diseased with PaL-4

Gross pathology was observed on the three fish diseased with pasteurellosis where isolate PaL-4 was collected. The fish had all been frozen and thawed, which made the identification of clinical signs more difficult. Multiple signs of sepsis, such as internal bleeding, oedema and skin ulcers, were found in these fish.

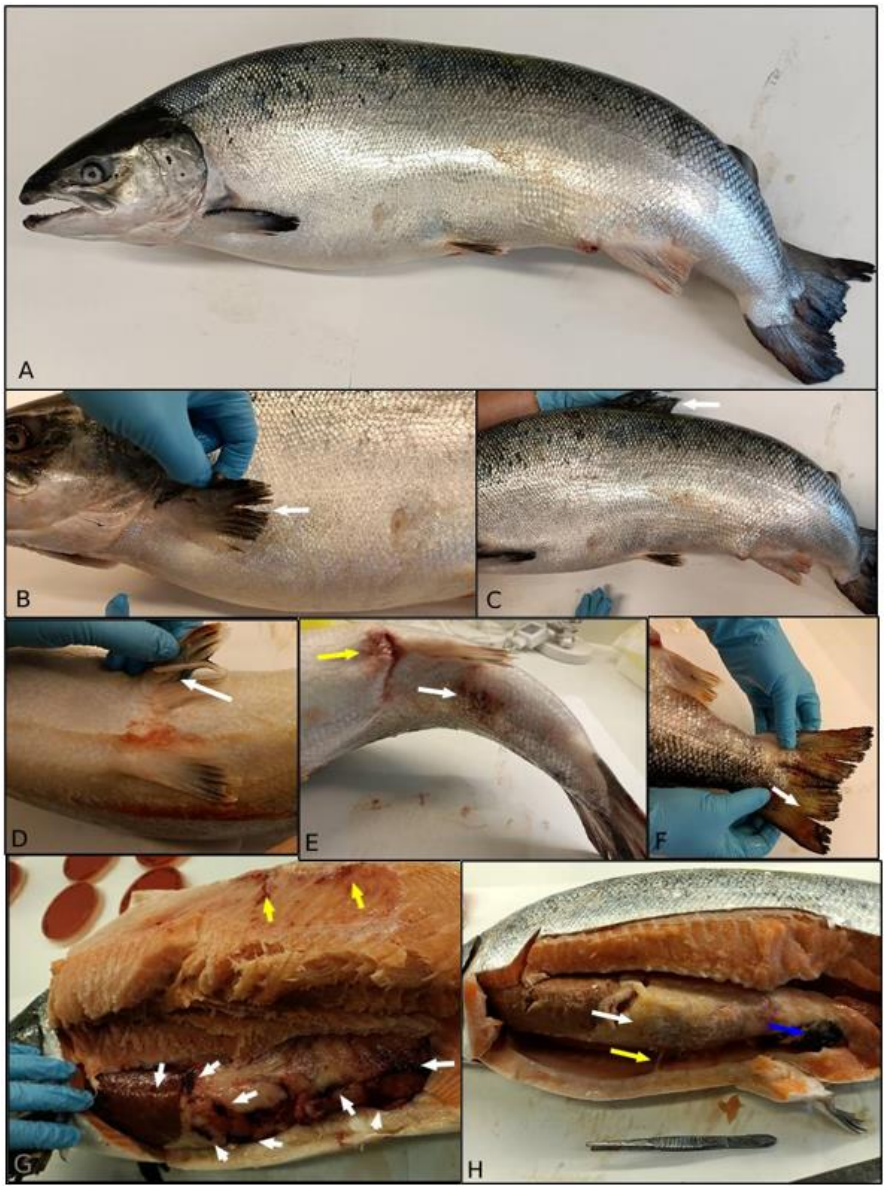


Figure 6 – Pictures from external and internal examination of diseased fish with pasteurellosis (PaL-4). Picture A displays an overview of the fish's left side. Picture B, C, D and F displays (white arrows) a frayed pectoral fin, dorsal fin, pelvic fin and caudal fin, respectively. In picture E show hemorrhage in the skin (white arrow) and around the anal opening (yellow arrow). Picture G displays hemorrhage in multiple internal organs (white arrows) and in the skeletal muscle (yellow arrows). Picture H displays abdominal adherence (yellow arrow) between the fatty tissue surrounding the pylorus caeca (white arrow) and abdominal muscle the wall tissue. The picture also displays a spleen of increased size (blue arrow).

Bacteria in pure culture after growth on BA from the head kidney of one of these fish where sanger sequenced, and analyzed with BLASTN (Zhang *et al.*, 2000; Morgulis *et al.*, 2008). The sequence showed to be 98% similar to *P. atlantica* strain NVI-9100 with the sequence ID CP074346.1.

## 3.2 - Gross pathology of fresh fish diseased with *P. atlantica* genomovar *salmonicida*.

Gross pathology was observed in the two salmon diseased with pasteurellosis examined at the field trip (Fig 7). These fish had not been frozen, making it easier to identify clinical signs of disease such as oedema, exophthalmia, and eye hemorrhage.

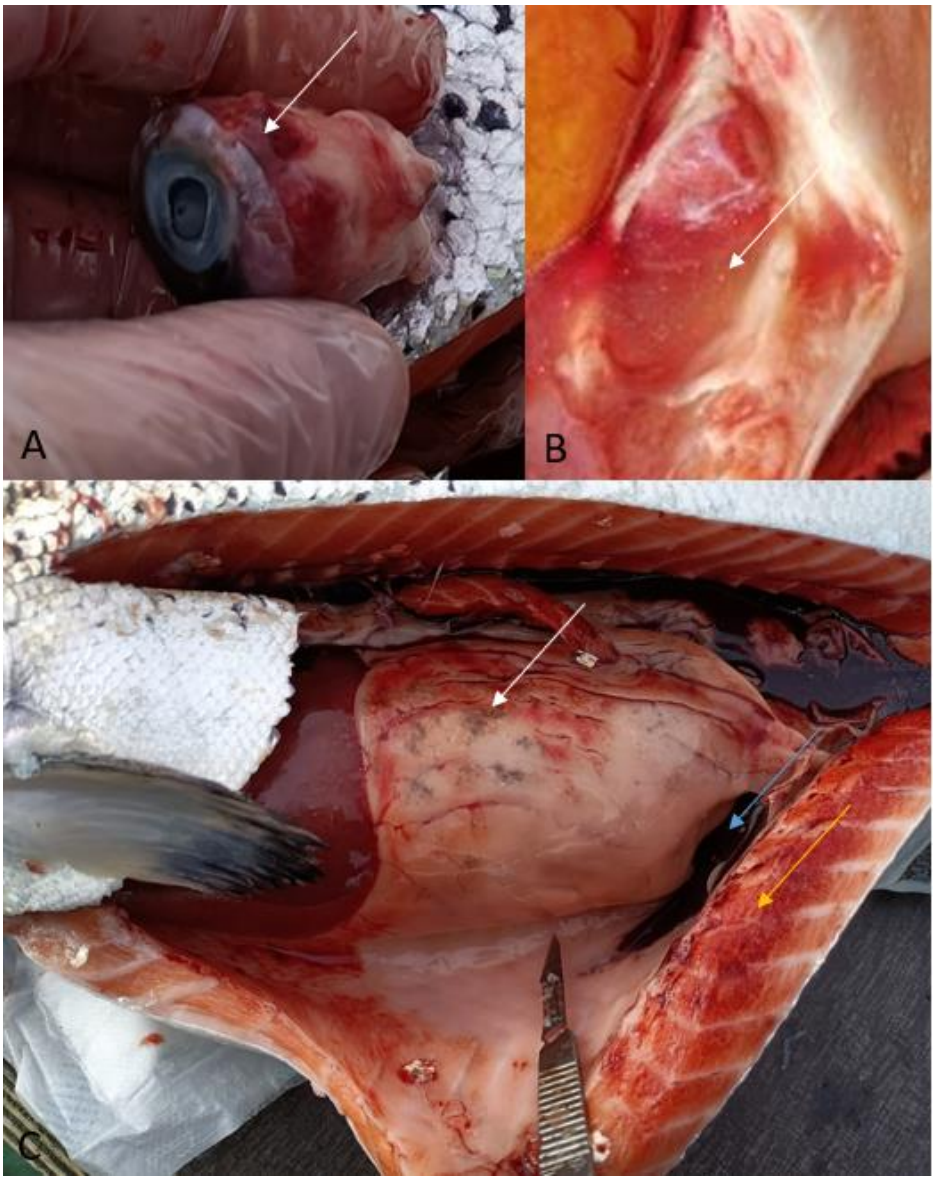


Figure 7 – Pictures from internal examinations of fresh fish diseased with pasteurellosis. Picture A displays hemorrhage (white arrow) around the eye of a fish with exophthalmia. Picture B displays oedema with white milky particles (white arrow) in the pericardium. Picture C displays the internal organs, with melanin spots (white arrow) on the fatty tissue surrounding the pylorus caeca, hemorrhage in the skeletal muscles (yellow arrow) and an enlarged spleen (blue arrow).

## 3.3 – Fish growth throughout the immunization period

Table 6 and Figure 8 both show the development of the fish average weight as measured at vaccination timepoints 1-3 and at the blood sampling. The control group was only measured during the first vaccination assay and during the blood collection assay. The fish group as a total grew from 127 g to 227 g average, resulting in a 1.17 % average daily growth rate for the 50 days the trial lasted. Excluding 8 days of starvation. No significant differences (appendix B) in growth were measured between the PaL-1, PaL-2, and Control group ($p>0.05$), however, the PaL-3 group had significantly lower growth than the three other groups ($p<0.032$).

Table 6 – Average (n=10) weight (±SD), length (±SD), and condition factor for all four groups during the vaccination and blood collection procedures.

| Procedure | Isolate | Average Weight (g) | SD weight (g) | Average Length (cm) | SD length (cm) | Condition Factor ($100*g/cm^3$) |
|---|---|---|---|---|---|---|
| Vaccination #1 | All | 127 | 13.9 | 21 | 1.0 | 1.37 |
| Vaccination #2 | PaL-1 | 170 | 24.1 | 23 | 1.1 | 1.38 |
| | PaL-2 | 175 | 19.9 | 23 | 0.81 | 1.35 |
| | PaL-3 | 148 | 19.9 | 22 | 1.1 | 1.33 |
| Vaccination #3 | PaL-1 | 191 | 28.6 | 24 | 1.0 | 1.38 |
| | PaL-2 | 201 | 21.3 | 25 | 0.89 | 1.35 |
| | PaL-3 | 168 | 26.5 | 24 | 1.3 | 1.24 |
| Blood sampling | PaL-1 | 234 | 32.6 | 26 | 0.93 | 1.39 |
| | PaL-2 | 238 | 25.4 | 26 | 1.4 | 1.30 |
| | PaL-3 | 196 | 33.0 | 24 | 1.6 | 1.36 |
| | Control | 243 | 28.5 | 26 | 1.4 | 1.35 |

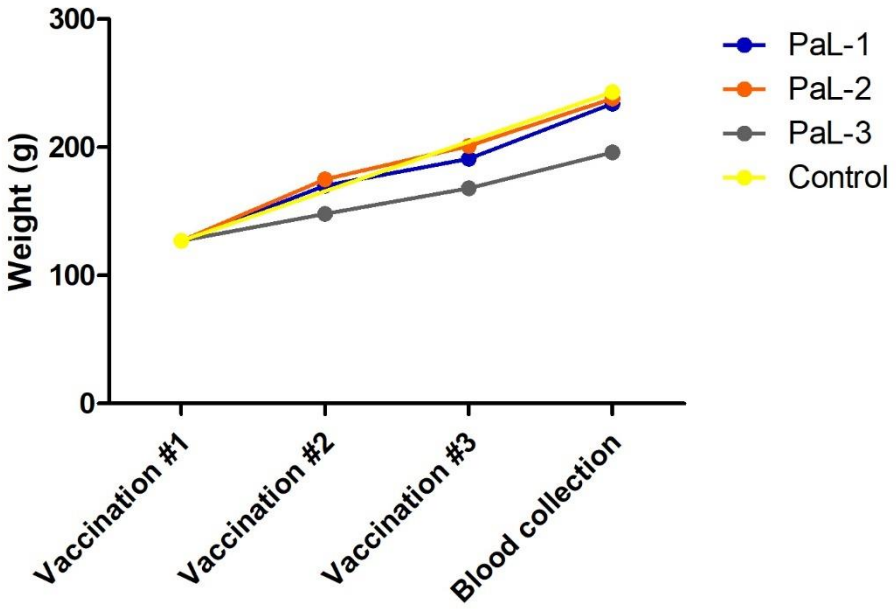


Figure 8 – Development of the fish's average weight throughout vaccination assay and blood collection assay.

Right after the blood collection, the fish's abdomens were opened to examine for side effect from the vaccine, like adherence and melanin spots. Only minor amounts adherence and melanin spots were observed.

## 3.4 – ELISA

Figures 9, 10 and 11 illustrate the results from the ELISA two-fold dilution series of sera collected from all three vaccination groups with the corresponding *Pasteurella* isolate used as antigen, as well as sera from the control group with all three isolates as antigen. A 1:400 dilution of sera was selected to examine cross-reactive properties for the antibodies produced against the different isolates, as the next dilution step's absorbance declines close to linearly. The control fish's sera have lower absorbance in the assay, as they lack specific antibodies against the bacterial isolates. Most of the sera from vaccinated fish have high absorbance, however a few individuals have absorbance close to the levels of the control fish's sera.

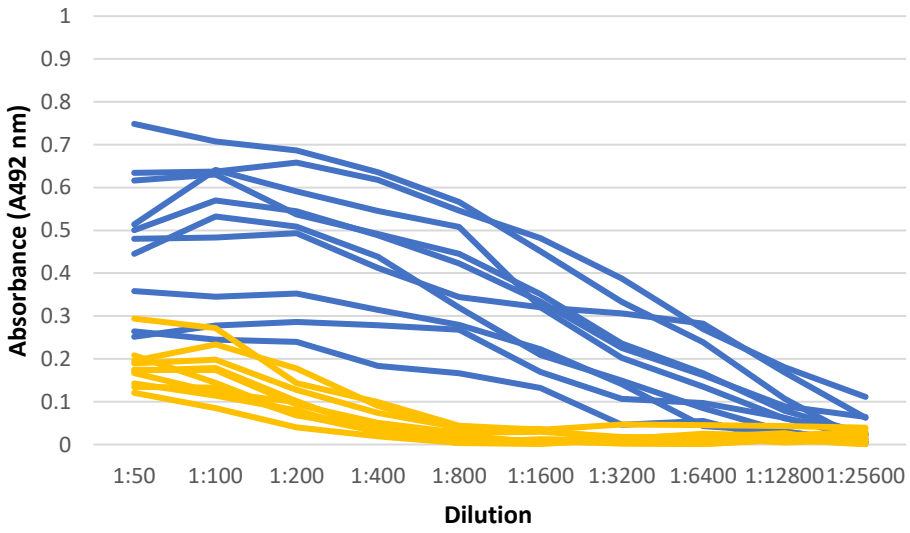


**Figure 9** – Dilution series of sera from PaL-1 vaccinated fish (blue lines) and control fish (orange lines) analyzed by ELISA, with PaL-1 bacteria as antigen.

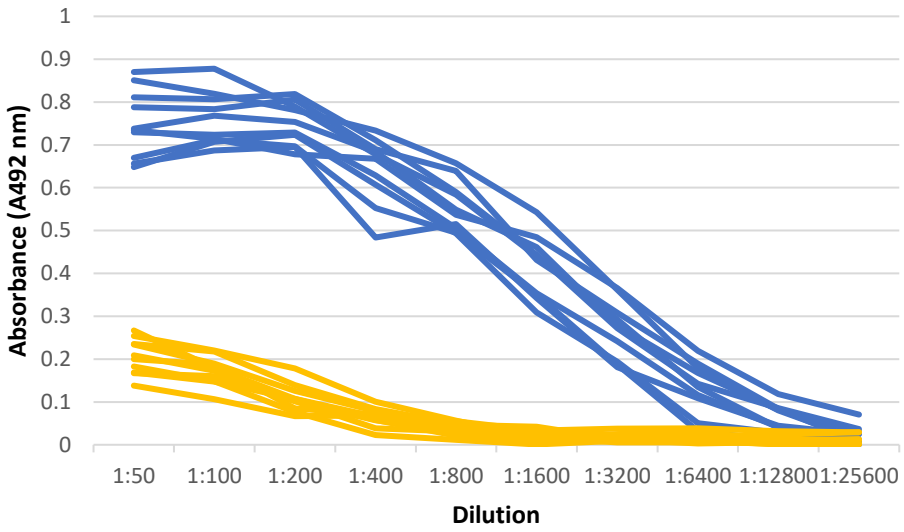


**Figure 10** – Dilution series of sera from PaL-2 vaccinated fish (blue lines) and control fish (orange lines) analyzed by ELISA, with PaL-2 bacteria as antigen.

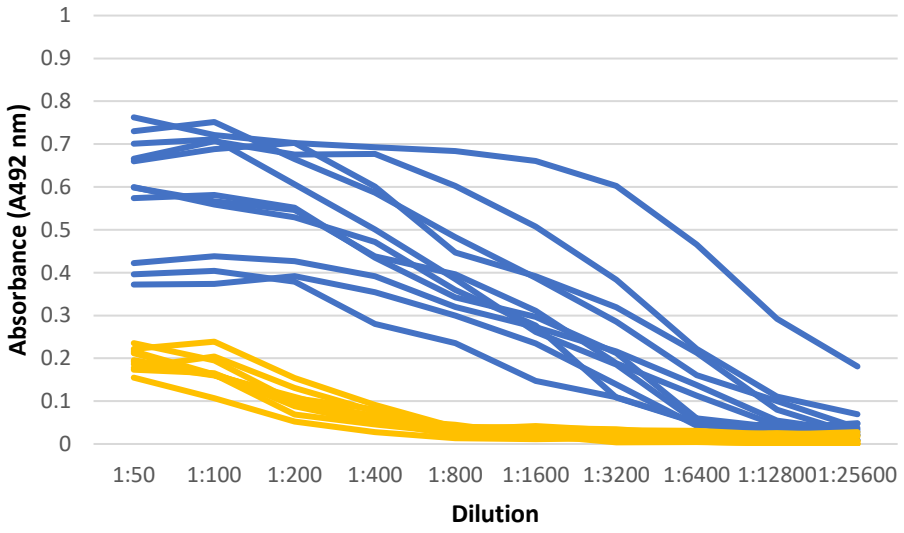


**Figure 11** – Dilution series of sera from PaL-3 vaccinated fish (blue lines) and control fish (orange lines) analyzed by ELISA, with PaL-3 bacteria as antigen.

The absorbance of all vaccine and control groups sera, diluted 1:400, when all four isolates have been used as antigen in the ELISA is presented in Figure 12. The antibodies produced after vaccination are clearly cross-reactive across the three genomovar *salmonicida* isolates, but only PaL-3 is slightly cross-reactive to the genomovar *cyclopteri* isolate (Figure 12 D). No significant difference was found between the salmon vaccine groups for each genomovar *salmonicida* antigen isolate used ($p > 0.05$), while the negative control groups where significantly lower compared to the vaccine groups for all genomovar *salmonicida* antigen isolates tested ($p < 0.05$). However, only the PaL-3 ($p = 0.0005$) vaccinated group and positive control group (sera from lumpsucker vaccinated with RK-1) ($p < 0.0001$) differ significantly from the negative control group, when RK-1 is used as antigen.

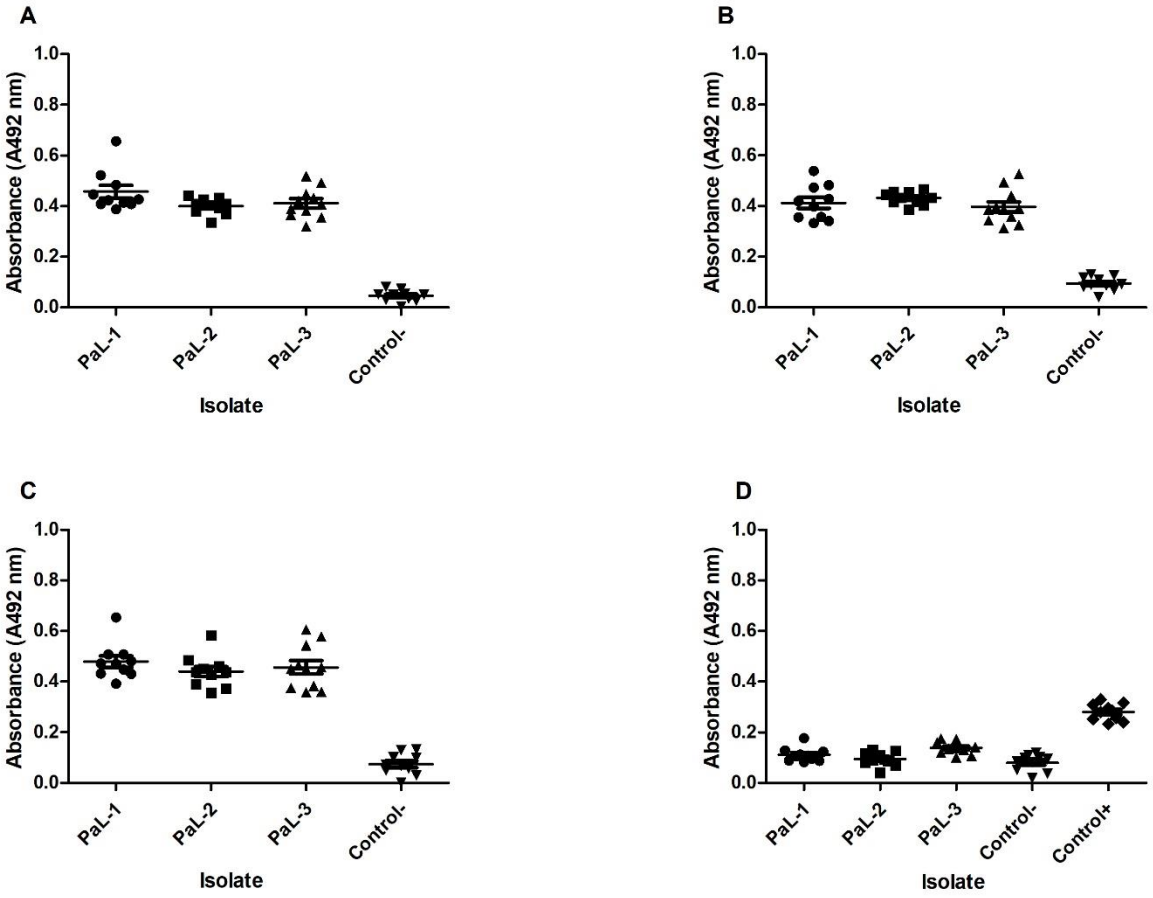


Figure 12 – Scatter plots showing reactivity of all vaccine and control sera, and all three *Pasteurella atlantica* genomovar *salmonicida* isolates as antigen, as well the RK-1 isolate, analyzed by ELISA. Plot A had PaL-1 as antigen, plot B had PaL-2 as antigen, plot C had PaL-3 as antigen, and plot D had RK-1 as antigen. Control- is sera from the non-vaccinated fish, and control+ is sera from lumpsuckers vaccinated with a similar vaccine based on the RK-1 isolate (Ellul *et al.*, 2019a).

## 3.5 – Silver staining and Western blot

Figure 13 displays the protein profile of five *P. atlantica* isolates presented through silver stained SDS-page gels. Note that the genomovar *salmonicida* isolates displays identical protein profiles, while the RK-1 isolate differ significantly. The strongest band (K) of the genomovar *salmonicida* were between 31.0 and 45.0 kDa in size, while the strongest band (B) of the RK-1 isolate was located between 45.0 and 66.2 kDa in size.

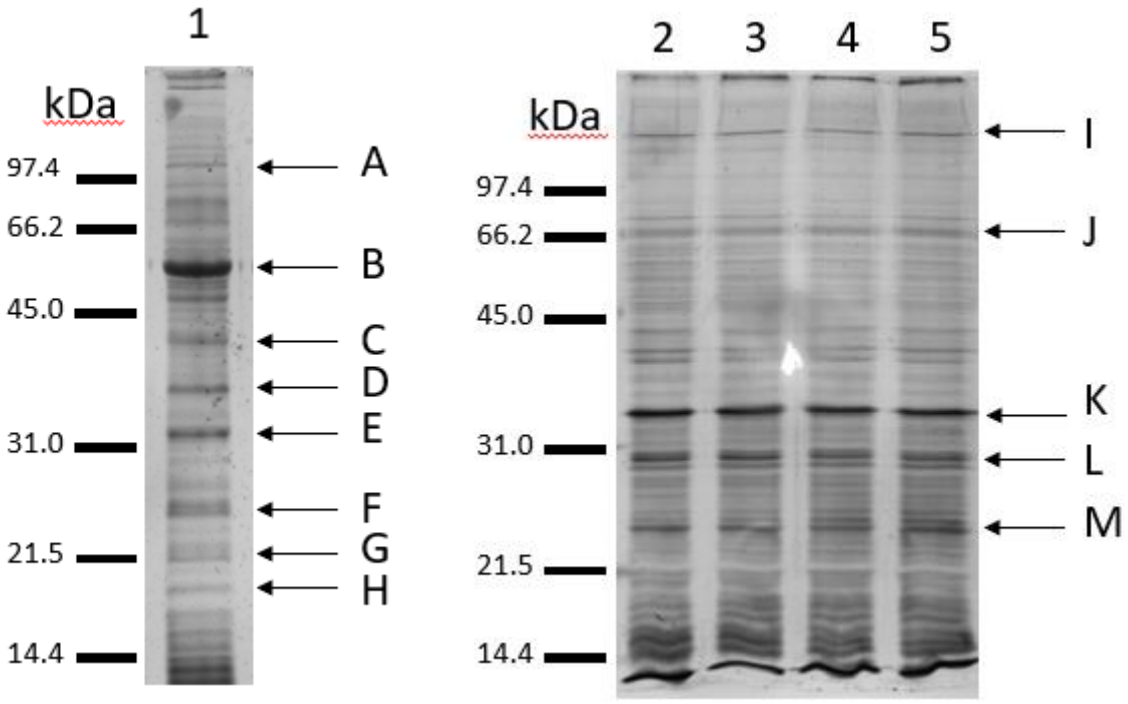


Figure 13 – Silver stained SDS-PAGE gels displaying protein profile of RK-1 (1), PaL-1 (2), PaL-2 (3), PaL-3 (4) and PaL-4 (5). Arrows marked A-H displays some of the most visible bands in the RK-1 gel, while the arrows marked I-M displays the most visible bands in the PaL-1 to PaL-4 gel. Appendix B for an approximation of the molecular weight of the most visible bands.

Figure 14 displays the same isolate proteins as figure 13, stained by antisera from vaccinated and control fish from the vaccination assay. The antibodies bound to proteins around roughly 75 kDa size for the genomovar *salmonicida* isolates, and to multiple distant bands between 25 and 150 kDa for the RK-1 isolate (Figure 14 B-D). The Pal-1, PaL-2 and Pal-3 antisera used gave similar results in the WB, but the PaL-3 sera (Figure 14 D) gave much weaker bands. The control fish's antisera gave no bands on the WB (Figure 14 A). No difference was observed when comparing sonicated (lane 6, 7 and 8) and non-sonicated (lane 2, 3,4 and 5) isolates in the WB assay.

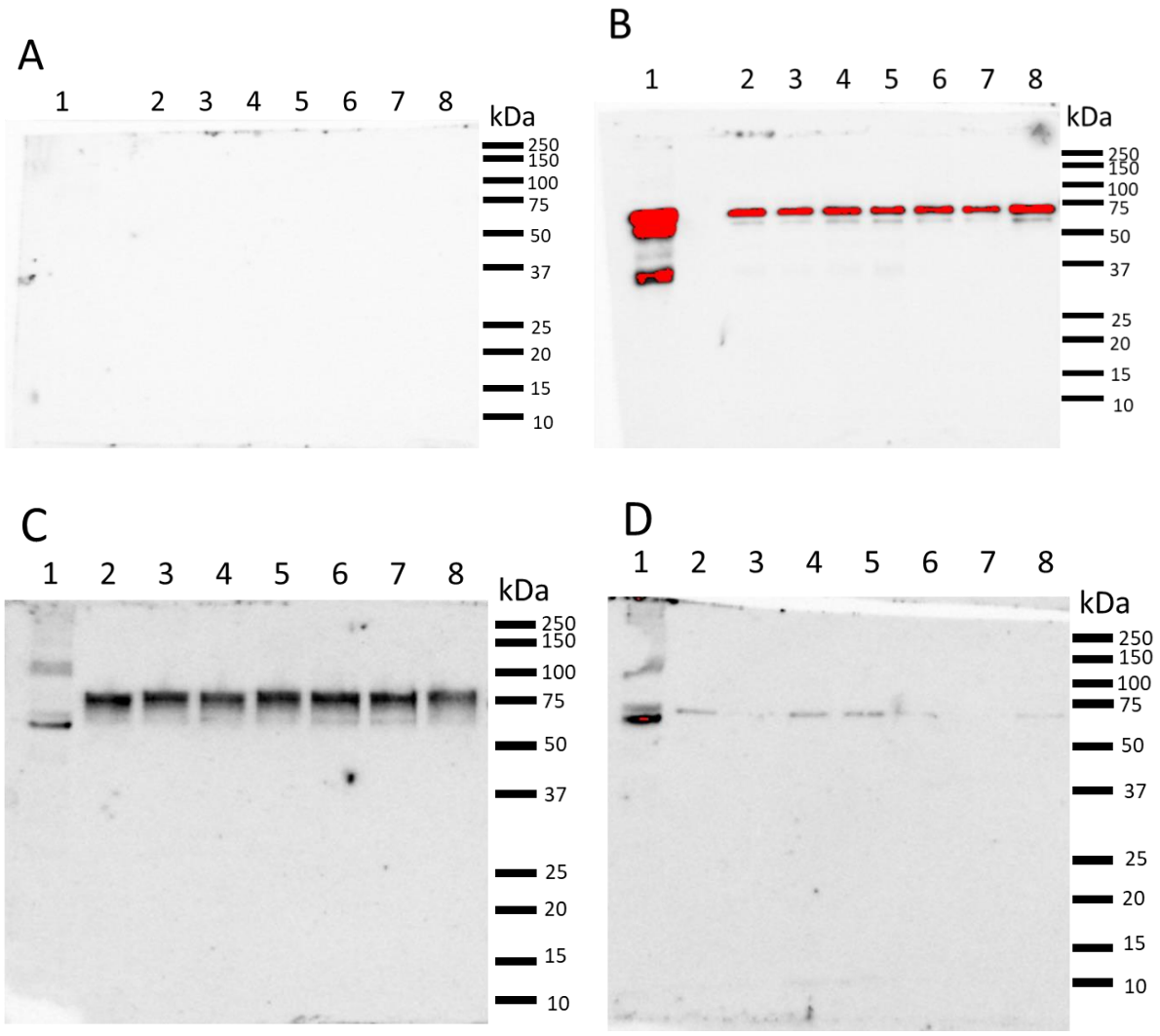


Figure 14 – Western blot with sera from control fish (A), PaL-1 (B), PaL-2 (C) and PaL-3 (D). Lanes 1-8 identify antigens as follows: RK-1 (1), PaL-1 (2), PaL-2 (3), PaL-3 (4), PaL-4 (5), PaL-1 (6), PaL-2 (7) and Pal-3 (8). Wells 2-5 includes non-sonicated bacterins as antigens, while well 1 and 6-8 includes sonicated bacterins. The figures display similar black and/or red bands around 75 kDa for the *Pasteurella atlantica* genomovar *salmonicida* and multiple different bands for the RK-1 isolate.

## 3.6 – Phylogenomic analysis

The heat map displays huge similarities between the genomovar *cyclopteri* isolates, between the genomovar *salmonicida* isolates, and between the *P. skyensis* isolates. The genomovar *salmonicida* isolates and the genomovar *cyclopteri* appeared to be more similar to each other, than to the *P. skyensis* isolates.

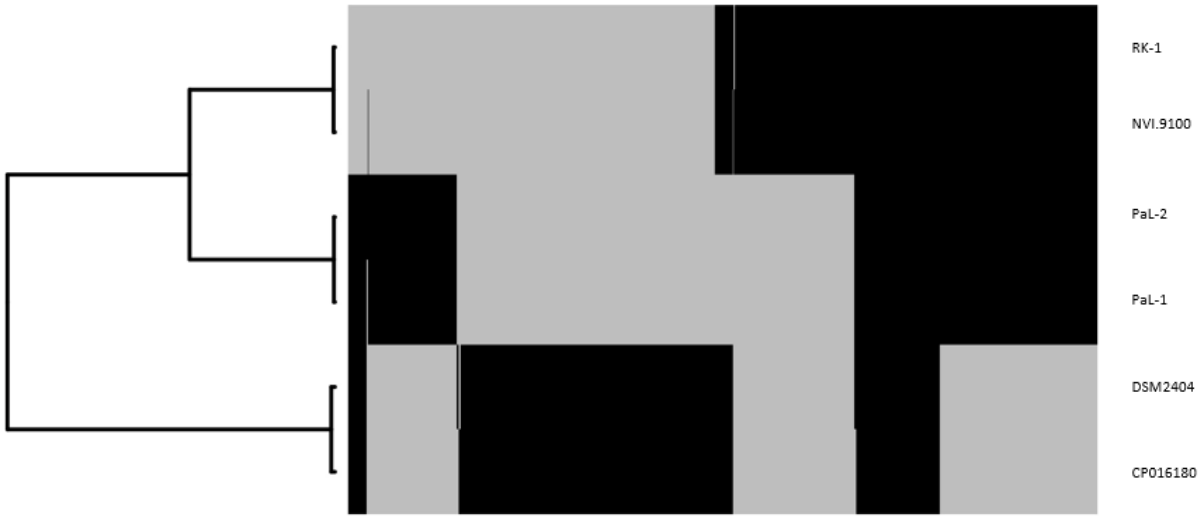


Figure 15 – Heat map and neighbor joining tree of two *Pasteurella atlantica* genomovar *cyclopteri* isolates, RK-1 and NVI 9100 (Ellul *et al.*, 2021), two *Pasteurella atlantica* genomovar *salmonicida* isolates, PaL-1 and PaL-2, and two *Pasteurella skyensis* isolates, DSM24204 (Foster *et al.*, 2000) and CP016180 (Hansen, Bojesen and Planet, 2016). Genes are considered to be similar if nucleotide sequences are ≥95% similar. Black and grey areas represent respectively the presence and absence of genes. The figure was made by co-supervisor Dr. Cyril Frantzen, ACD Pharma.

The Neighbor joining tree also displays that the *P. atlantica* isolates are more similar to each other, then to the *P. skyensis* isolates, and that the isolates are more similar to each other within the genomovar, than across it. The genomic variation within each genomovar, and within *P. skyensis*, showed to be very low. Nearly half of the common genes showed to differ between across the genomovars.

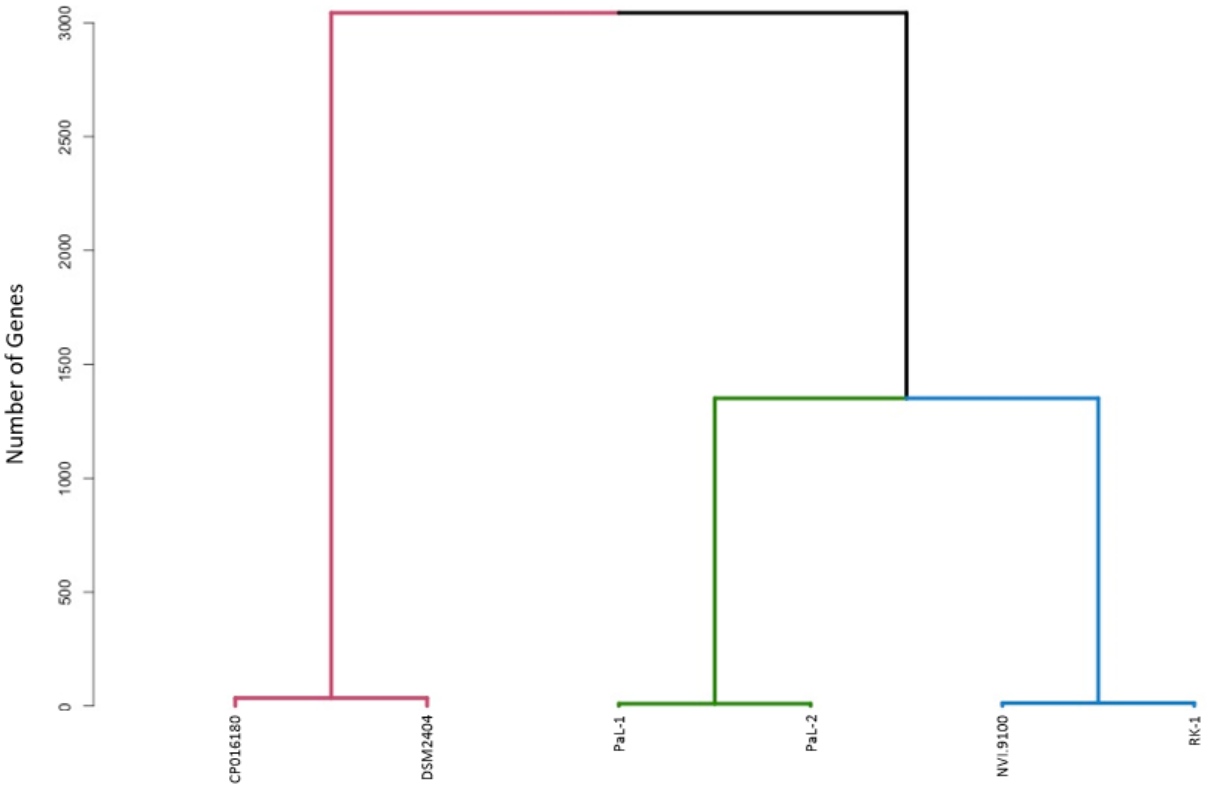


Figure 16 – Neighbor joining tree of two *Pasteurella atlantica* genomovar *cyclopteri* isolates, RK-1 and NVI 9100 (Ellul *et al.*, 2021), two *Pasteurella atlantica* genomovar *salmonicida* isolates, PaL-1 and PaL-2, and two *Pasteurella skyensis* isolates, DSM24204 (Foster *et al.*, 2000) and CP016180 (Hansen, Bojesen and Planet, 2016). Genes are considered to be similar if nucleotide sequences are ≥95% similar. The x axis shows number of genes that differ between the isolates, excluding genes that's not common against any of the isolates. The figure was made by co-supervisor Dr. Cyril Frantzen, ACD Pharma.

# 4 – Discussion

The aims of this study were to analyze specific antibody responses towards *P. atlantica* genomovar *salmonicida*. To produce antisera, oil-based vaccines against three isolates of *P. atlantica* genomovar *salmonicida* were produced and used to vaccinate Atlantic salmon. The harvested antisera were examined for specific and cross reactivity against *P. atlantica* isolates. The protein profiles of the isolates were examined by silver stained SDS-PAGE gels and immunogenic components visualized by WB.

The antisera produced had high OD in the ELISA assays, indicating that the immune responses of the fish were substantial. There were some individual variations in antibody responses, as expected, but almost all sera from vaccinated fish had OD higher than that of sera from the non-vaccinated control fish. Only two individuals, injected with the PaL-1 vaccine, did not respond to the vaccination by production of specific antibodies. The individual variations in the current assay is comparable to that of similar research using *P. atlantica* bacterins (Ellul *et al.*, 2019a).

The antisera produced were highly cross-reactive across the *P. atlantica* genomovar *salmonicida* isolates, with no significant difference in OD measured independent of which genomovar *salmonicida* isolate the antibodies originally targeted. This suggest that the bacteria's surface immunogenic epitopes are highly similar. Moreover, the cross-reaction to the RK-1 isolate appeared to be weak when analyzed by ELISA. This indicates that the surface epitopes of this isolate are quite different from that of the genomovar *salmonicida* isolates. The heat map and the SDS-PAGE gels further substantiates that isolates belonging to genomovar *salmonicida* are very similar in their protein profile, both when it comes to potential protein expression, as shown in the heat map, and expressed proteins, while they differ significantly from the RK-1 isolate and from *P. skyensis*.

It was also found that the salmon antisera targeted proteins of roughly the same molecular mass, at ~ 75 kDa, from all genomovar *salmonicida* isolates when analyzed by WB. This substantiates some of the findings in the ELISA assays: that the bacteria's immunogenic epitopes are highly similar. However, the salmon antisera targeted proteins of different molecular mass on the RK-1 isolate a ~ 37 kDa protein was targeted by the anti-PaL-1 sera and a ~ 60 kDa protein by the anti-PaL-2 and anti-Pal-3 sera. This tells us that the antibodies from the salmon sera still targets proteins of the RK-1 isolate, but that the proteins are of a different molecular mass. This also show the diversity of antibodies present in the salmon sera after vaccination. Furthermore, it substantiates that there is cross-reactivity from the antibodies from the salmon sera to the antigens of the RK-isolate, even though this cross-reactivity proved to be weak and/or insignificant when analyzed by ELISA.

Several clinical signs of pasteurellosis were found in the fish diseased with PaL-4 and the fish from the field trip diseased with pasteurellosis. Exophthalmia, hemorrhage around the eye, edema with white milky particles in the pericardium, petechial bleedings in multiple internal organs and skeletal muscle with sepsis and an increased spleen are all characteristic clinical signs found, that correspond with previously described *P. atlantica* genomovar *salmonicida* infections (Legård and Strøm, 2020; Sommerset *et al.*, 2022). These clinical signs fit what is found in commercial fish farms with elevated mortality due to outbreaks of pasteurellosis.

After vaccination, the fish grew from 127 g to 227 g on average for the 58 days of the trial, resulting in a 1.17% daily growth rate. According to the company manufacturing the fish feed, the fish should have had a daily growth rate of 1.89-2.09% (Skretting AS, 2012). However, a lower growth rate is to be expected as stressors, such as vaccination assays, are associated with a lower growth rate (Campbell *et al.*, 2017), and reduced growth rate is also commonly observed when rearing fish in small tanks for fish trials. The PaL-3 vaccinated fish had a significantly lower growth rate than the other groups ($p > 0.032$, appendix B) and had a growth rate of 0.87%. A difference in the starting weight cannot be disregarded as the causation of this difference in growth rate, as the last 14 fish of the first vaccine trial were not weighed, including all 11 fish vaccinated with the PaL-3 vaccine. However, there were little variation in the starting weight of the fish, and thus the vaccine might have had an influence difference in daily growth rate. This difference in growth rate could be due to other factors such as selection procedure of fish for vaccination, the amount of adjuvant added to the vaccine, or stressors like handling of the fish. These factors should ideally be random and equally distributed across all fish groups, however we cannot exclude this as an explanation.

## 4.1 – ELISA

Through the ELISA assays, it was displayed that almost all the fish responded to the vaccine by producing specific antibodies against the vaccine isolate. There were some individual variation, but it showed to be within what is common for similar experiments (Romstad *et al.*, 2012a; Rønneseth *et al.*, 2015; Ellul *et al.*, 2019a). The OD in the assays proved to be high, and significantly higher measurements were recoded from sera from vaccinated fish compared to the control fish, and thus proving the production of specific antibodies post vaccination.

The antibodies produced were clearly cross-reactive across all three genomovar *salmonicida* isolates. This indicates that a vaccine based on either one of these three isolates, would induce production of antibodies reactive towards all three isolates. However, Ellul *et al.* (2019a) did make a vaccine based on the RK-1 isolate, and got a sufficient antibody response, but insufficient protection against

pasteurellosis in lumpsuckers. So even if there is a sufficient antibody response across all three genomovar *salmonicida* isolates analyzed in this thesis, it does not mean that the vaccine would provide sufficient protection against pasteurellosis. To test this an infection challenge would have to be performed.

When the RK-1 isolate was used as antigen in the ELISA assays, we found that the antibodies produced in lumpsuckers against the RK-1 isolate provided high OD values, but the sera from salmon vaccinated with genomovar *salmonicida* provided OD values similar to what was observed for non-vaccinated controls. This is even though Ellul *et al.* (2019a) only diluted the lumpsucker sera 1:50, while all sera in this thesis, including the lumpsucker's sera, were diluted 1:400. It is not uncommon for salmon to produce more antibodies than lumpsuckers after similar vaccine trials (Rønneseth *et al.*, 2007; Ellul *et al.*, 2019a). Only the PaL-3 vaccinated fish produced sera with somewhat significantly higher ($p = 0.0005$) OD than that of the negative control salmon's sera, but still low enough for the cross-reaction to be considered as weak.

This indicates that the antisera produced from the PaL-3 vaccinated fish are cross-reactive across the two genomovars, but as the optical density was low, the cross-reactivity is likely to be weak, and thus would probably not give protection against both genomovars. Moreover the genomovar *cyclopteri* isolates might not be able to infect salmon, but both lumpsuckers and salmon can be infected by the genomovar *salmonicida* isolates (Sandlund *et al.*, 2021). And thus, a strong cross-reaction would probably not be necessary to protect the salmon.

## 4.2 - Silver staining SDS-PAGE gels, and Western blot

Through the silver staining assay, it was shown that all four genomovar *salmonicida* isolates had protein profiles that seems to be identical. This indicates that the isolates are similar in their expression of surface proteins, at the given growth conditions, and thus a vaccine based on one of the isolates would be likely to protect the fish against all four isolates. However, the protein profile of the RK-1 isolate showed to differ significantly from the genomovar *salmonicida* isolates, and thus the same cannot be said for this isolate. Even though these genomovars are classified as the same species, their protein profiles were shown to not be similar.

The most prominent protein bands of the RK-1 isolate are marked A-H in Figure 14, and the most prominent protein bands of the genomovar *salmonicida* isolates are marked I-M. By comparison, the marked protein bands of the genomovar *salmonicida* isolates and the genomovar *cyclopteri* isolate do not appear to be of the same molecular weight. Appendix B displays an approximation of the molecular

weight of the most visible bands, and an overview of the closes similar band. Band F and M seems to be two closest bands, at about 25 and 24 kDa respectively.

Through the WB assay, it was shown that the salmon antisera targeted proteins of about 75 kDa for the genomovar *salmonicida* isolates, and multiple bands between 37 and 150 kDa for the RK-1 isolate. This means that even though there is cross reactivity to the RK-1 isolate, the antibodies target proteins of different sizes for the isolates. Through the ELISA assays, it was shown that only the PaL-3 isolate had cross-reaction to the RK-1 isolate, and even PaL-3 had low OD values. In the WB assay, sera from all three genomovar *salmonicida* vaccine groups showed cross-reactivity towards the RK-1 isolate, and the intensity of the bands identified seems to be similar. One explanation to this, is that the proteins in the WB are denatured, while they're not in the ELISA assays, which can affect the antibodies ability to bind to their target epitopes (Karlsson *et al.*, 1989; Forsström *et al.*, 2015).

The results from the WB assay indicates that the salmon antisera were cross-reactive to the RK-1 isolate, while the ELISA assay indicated that this cross-reactive property is very weak. This could have multiple explanations: Firstly, it could be the case that there was added a higher concentration of antibodies (1:100) to the WB assay, and thus another reagent of the assay was the bottleneck of all the reactions, and thus the bands turned out to be of the same intensity. Also, only one fish's sera from each vaccination group were used as antisera in the WB assay, and these individual fish all showed to have above average measurements of OD in the ELISA assays when exposed to the RK-1 isolate as antigen. A third possible explanation is that WB is a more sensitive assay, and thus can more easily display this cross-reactivity. It is already proven from other research that WB is a more sensitive approach than ELISA (Karlsson *et al.*, 1989). However, keep in mind that WB is not a quantitative measurement, as the ELISA assays are, but the difference in results were significant enough that this had to be addressed. Another important factor that may contribute to the difference in results is the issue of protein denaturation by using β-mercaptoethanol in the samples loaded on the nitrocellulose membranes for WB as discussed earlier.

Some of the targeted proteins of the RK-1 isolate are similar in size to the targeted proteins of the genomovar *salmonicida* isolates in the WB, so it is possible that the antibodies also target proteins that are similar between the RK-1 and the genomovar *salmonicida* isolates. As illustrated by the heat map, about half of the displayed genes were common between the genomovar *salmonicida* isolates and the genomovar *cyclopteri* isolates, and thus, it would not be unreasonable to predict that similar OMPs may be expressed. Therefore, antibodies that target proteins of the genomovar *salmonicida* isolates, may also target proteins of the genomovar *cyclopteri* isolates.

The different antisera used in the WB assay provided different intensity of the bands of immunogenic proteins that appeared. Firstly, antibodies in the sera from the control fish did not bind, which was as expected as the fish had not been immunized with the isolates, and thus should not have antibodies reactive against the isolates. The antisera from the PaL-3 vaccinated fish gave much weaker bands than that of the PaL-1 and PaL-2 vaccinated fish. This could indicate that the PaL-3 vaccinated fish produced less or weaker binding antibodies against the isolates, however no significant difference in antibody response was detected in the ELISA assay, indicating that a larger fraction of the antibodies may be directed towards conformational epitopes as the samples have been treated with β-mercaptoethanol, which can change the epitopes (Forsström *et al.*, 2015). Also, only sera from one fish from each vaccination group were chosen for the WB assay, and the fish with the highest specific reaction from the ELISA assays was chosen for this assay. This can give the impression of a stronger reaction in the WB assay, than what an average fish's sera from each vaccination groups could have performed.

The WB assay targets proteins, so even highly immunogenic carbohydrates would not be shown in such an assay (Burnette, 1981). Therefore, an explanation as to why the immunosera from the PaL-3 vaccinated fish gave weaker bands, could be that the PaL-3 isolates have more immunogenic carbohydrates than the PaL-1 and PaL-2 isolates. A vaccine based on the PaL-3 isolate could still be effective, even if the antibodies targeted surface carbohydrates, rather than surface proteins, as both can be essential for infection (Wizemann, Adamou and Langermann, 1999). This would also explain why the ELISA assay showed no significant difference in OD between the genomovar *salmonicida* isolates, as the ELISA assay could target both proteins and carbohydrates as antigens (Engvall and Perlmann, 1971).

We do not know the structural nature of the epitopes targeted by the antibodies in the immunosera. Conformational epitopes differ from linear epitopes by being folded into three dimensional structures to be functional, and the preparation of antigens before the WB assays can change these epitopes (Forsström *et al.*, 2015). These changes can include an unfolding of the epitopes, changing its structure leading to loss of recognition and affinity by the specific antibodies produced. Additionally, unfolding can expose epitopes that can be targeted by the antisera tested. This means that the positive results of cross-reactivity from the WB of the salmon sera to the RK-1 isolate, may potentially be a false positive. However, it could also be that the preparation of antigens for the ELISA assays have changed the epitopes of the antigen, and thus provoke a false weak cross-reactive result.

The PaL-1, PaL-2 and PaL-3 vaccinated fish all produced antisera with similar results, and thus further substantiates the results of the SDS-PAGE gels, that these isolates are highly similar in expressed proteins, especially for the immunogenic proteins.

Moreover, the PaL-3 vaccinated fish gave weaker bands with the WB assay, and more cross-reactivity in the ELISA assays. The PaL-3 vaccinated fish also grew significantly less in weight and length than the other groups, and thus the PaL-1 and PaL-2 isolate would probably be more useful as a candidate for a commercially available vaccine. However, as already discussed, this could have explanations, so that the PaL-3 isolate should still not be discarded as a potentially candidate for vaccine development.

## 4.3 - Phylogenomic analysis

A heat map of two genomovar *cyclopteri* (including RK-1), two genomovar *salmonicida* (PaL-1 and PaL-2) and two *P. Skyensis* isolates was compiled to reveal similarities and differences in the genomes of these isolates. The map proved that there was little difference between isolates within the same genomovar. The two genomovars showed to be more similar to each other, than to the *P. Skyensis* isolates. However, the difference between the two genomovars is still significant, and substantiates the findings in the SDS-PAGE gels: that there are significant differences in the proteins of the two genomovars. These findings are as expected as the two genomovars are more closely related to each other, than to *P. skyensis*, and difference in proteins between the two genomovars would be expected, as they infect different host species.

The heat map displays that nearly half of the genes displayed showed to differ across the two genomovars. This tells us that there is a great difference in potential proteins across these two genomovars, and the SDS-PAGE gels substantiates that this is the case when examining the protein profile of the expressed genes. We could also find differences in the WB analysis across these genomovars, indicating that these differences also apply for the most immunogenic genes.

The small differences within each genomovar were also interesting, as it suggests that each genomovar is a homologous group. The SDS-PAGE gels and WB assays also affirm this for the genomovar *salmonicida* group.

## 4.4 – Limitation of work, broader implications, and directions for future research

In this study antibody response post vaccination with bacterins of *P. atlantica* genomovar *salmonicida* was studied in Atlantic salmon. The antisera were used to analyze the production of specific antibodies and examine cross-reactive properties. The antibody response and the cross-reactive properties of the antibodies proved to be satisfactory, however, we do not know whether the antibodies produced are protective in nature. To verify protection against pasteurellosis a controlled infection challenge of vaccinated fish would have to be provided, in order to calculate the vaccine's RPS. Ellul *et al.* (2019a) did test this with a similar vaccine based on the RK-1 isolate, and got a sufficient antibody response, but insufficient protection against pasteurellosis in lumpfish. As genomovar *cyclopteri* and genomovar *salmonicida* are closely related, it would not be too surprising if it would be the same case for salmon.

In this thesis, we demonstrate that the protein profile of these two genomovars differ, both in the potential proteins of the bacteria (heat map) and the expressed proteins (silver-stained protein gel), the phylogenomics differ, and that the antisera produced bind to proteins of different molecular weights.

The silver stained SDS-PAGE gels displayed that there was little to no difference in the protein profile of the genomovar *salmonicida* isolates. The WB displayed that the antibodies targeted proteins of roughly the same molecular mass, and through the ELISA of the immune sera we found that there was no significant difference in OD based on which isolate the fish was vaccinated with. All of this indicates that the isolates are largely similar, and that a vaccine that provide sufficient protection against infection by one of these isolates, probably would also protect against all these isolates. This is good news, as development of multicomponent vaccines becomes more difficult the more pathogens that are to be included in a vaccine (Reglinski *et al.*, 2016). Indicating that only one *P. atlantica* genomovar *salmonicida* isolate would have to be included in a vaccine to protect salmon against pasteurellosis.

Also, the high OD values obtained from the ELISA assays is good news, as the protection obtained from vaccination often correlates to the amount of antibodies produced (Romstad *et al.*, 2012a, 2012b). In the current trial, salmon were vaccinated three times to produce as high amounts of antibodies as possible and will thus not likely reflect antibody levels produced after one injection using the same antigen concentration in the vaccine. If correlation between antibody response and protection against pasteurellosis can be confirmed, this knowledge can be used to reduce the number of fish needed in efficacy and batch tests for commercializing vaccines against pasteurellosis in salmon.

As already mentioned, the PaL-1 and PaL-2 vaccinated fish grew the most and produced stronger bands in the WB assay, compared to the PaL-3 vaccinated fish. The PaL-2 isolate was the one with the least

variation in OD through the ELISA assays, but more research will be needed to verify if this will have an impact on the selection of vaccine isolate.

The WB assay displayed the molecular mass of the proteins that the antibodies bound to. However, the identity of these proteins is still unknown, as well as the number of different proteins present in each band. It would be useful for vaccine development to identify these proteins and find whether they are essential for infection or not. Further analysis of these proteins would be useful.

Also, through the heat map, it was displayed how many similar genes the six isolates had, but the function of the genes remains unknown. Further analysis of the identity and function of these common genes would also be helpful for vaccine development.

It would also be advantageous to identify immunogens of the genomovar *salmonicida* isolate, as they could be directly targeted as candidates for vaccine development. The WB assay performed helped to identify the molecular mass of some antigens believed to be the most immunogenic of the isolates, however, some of the antigens not targeted by this vaccine, could still be targeted in a subunit vaccine if further research could identify an essential antigen not targeted by this vaccine.

Developing and testing vaccines based on other vaccine technologies would also be helpful. Nucleic acid vaccines have proven to induce more T-cell mediated immunity than B-cell mediated immunity, which could increase immunity for some types of infections (Vogel and Sarver, 1995; Murphy and Weaver, 2017). It would be interesting to see if this was the case with genomovar *salmonicida* isolates, or if the classical vaccines are the best approach to avoid pasteurellosis. As mentioned in the introduction: Members of the family *Pasteurellaceae* may have been found intracellularly in phagocytic immune cells of Atlantic salmon, and this is also suspected to be the case in lumpsucker infected with *P. atlantica* genomovar *cyclopteri* (Jones and Cox, 1999; Ellul *et al.*, 2019b; Legård and Strøm, 2020). If this turns out to be the case for genomovar *salmonicida*, then the bacterium would be able to hide from antibodies, and thus reducing the effect B-cell mediated immunity. However, T-cells can neutralize such infected cells, and that would make nucleic acid vaccines a better choice of strategy.

# 5 – Conclusions

Several conclusions answering the initial aims of the thesis are presented.

- Vaccination of salmon using bacterins of P. *atlantica* genomovar *salmonicida* induced production of specific antibodies.
- Cross-reactive properties of antibodies raised against the different isolates were verified.
- Antibodies raised against P. *atlantica* genomovar *salmonicida* had only little cross-reactive properties for binding to P. *atlantic*a genomovar *cyclopteri* when examined by ELISA.
- The salmon isolates had identical protein profiles and were significantly different from the lumpsucker isolate.
- The salmon antisera produced bound to proteins of similar molecular weight of the genomovar *salmonicida* isolates, but different for the genomovar *cyclopteri* isolate when analyzed in WB.

# 6 – References

Abterra Biosciences (2020) Major antibody isotypes. Available at: https://abterrabio.com/2020/05/25/antibody-reference-guide/ (Accessed: 1 April 2022).

Alarcón, M., Gulla, S., Røsæg, M. V., Rønneseth, A., Wergeland, H., Poppe, T. T., Nilsen, H., Colquhoun, D. J. (2016) 'Pasteurellosis in lumpsucker *Cyclopterus lumpus*, farmed in Norway', Journal of Fish Diseases. John Wiley & Sons, Ltd, 39(4), pp. 489–495. doi: 10.1111/JFD.12366.

Bone, Q. and Moore, R. H. (2008) 'Biology of Fishes', Third Edit. Edited by E. Owen, K. Lyons, and K. Henderson. Abingdon: Taylor & Francis Group.

Brudeseth, B. E., Wiulsrød, R., Fredriksen, B. N., Lindmo, K., Løkling, K. E., Bordevik, M., Steine, N., Klevan, A., Gravningen, K. (2013) 'Status and future perspectives of vaccines for industrialised fin-fish farming', Fish & Shellfish Immunology. Academic Press, 35(6), pp. 1759–1768. doi: 10.1016/J.FSI.2013.05.029.

Burnette, W. N. (1981) '"Western Blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A', Analytical biochemistry, 112(2), pp. 195–203. doi: 10.1016/0003-2697(81)90281-5.

Campbell, N. A., Urry, L. A., Cain, M. L., Wasserman, S. A., Minorsky, P. V., Reece, J. B. (2017) 'Biology: A Global Approach, Global Edition', in Biology: A Global Approach, Global Edition. Eleventh E. Pearson Education, p. 510.

Collins, M. D., Wallbanks, S., Lane, D. J., Shah, J., Nietupski, R., Smida, J., Dorsch, M., Stackebrandt, E. (1991) 'Phylogenetic analysis of the genus *Listeria* based on reverse transcriptase sequencing of 16S rRNA', International Journal of Systematic Bacteriology. Microbiology Society, 41(2), pp. 240–246. doi: 10.1099/00207713-41-2-240/CITE/REFWORKS.

Cusabio Technology LLC (2021) 'Cross-reactivity of Antibody: Beneficial or Harmful?' Available at: https://www.cusabio.com/c-21045.html (Accessed: 1 April 2022).

Directorate of fisheries (2021) 'Atlantic salmon, rainbow trout and trout - Grow out production'. Directorate of fisheries. Available at: https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Laks-regnbueoerret-og-oerret/Matfiskproduksjon (Accessed: 15 November 2021).

Ellul, R. M., Bulla, J., Brudal, E., Colquhoun, D., Wergeland, H., Rønneseth, A. (2019a) 'Protection and antibody reactivity in lumpsucker (*Cyclopterus lumpus* L.) following vaccination against *Pasteurella* sp', Fish & shellfish immunology. Academic Press, 95, pp. 650–658. doi: 10.1016/j.fsi.2019.11.016.

Ellul, R. M., Walde, C., Haugland, G. T., Wergeland, H., Rønneseth, A. (2019b) 'Pathogenicity of *Pasteurella* sp. in lumpsuckers (*Cyclopterus lumpus* L.)', Journal of fish diseases. Blackwell Publishing Ltd, 42(1), pp. 35–46. doi: 10.1111/jfd.12905.

Ellul, R. M., Kalatzis, P., Frantzen, C., Haugland, G., Gulla, S., Colquhoun, D., Middelboe, M., Wergeland, H., Rønneseth, A. (2021) 'Genomic analysis of *Pasteurella atlantica* provides insight on its virulence factors and phylogeny and highlights the potential of reverse vaccinology in aquaculture', Microorganisms. MDPI AG, 9(6). doi: 10.3390/MICROORGANISMS9061215.

Engvall, E. and Perlmann, P. (1971) 'Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G', Immunochemistry (1965), 8(9), pp. 871–874. doi: 10.1016/0019-2791(71)90454-X.

Forsström, B., Bisławska, B., Rockberg, J., Danielsson, H., Bohlin, A., Uhlen, M. (2015) 'Dissecting Antibodies with Regards to Linear and Conformational Epitopes', PLOS ONE. Public Library of Science, 10(3), pp. e0121673–e0121673. doi: 10.1371/journal.pone.0121673.

Foster, G., Ross, H., Malnick, H., Willems, A., Hutson, R., Reid, R., Collins, M. (2000) 'Phocoenobacter uteri gen. nov., sp. nov., a new member of the family Pasteurellaceae Pohl (1979) 1981 isolated from a harbour porpoise (*Phocoena phocoena*)', International Journal of Systematic and Evolutionary Microbiology. Society for General Microbiology, 50(1), pp. 135–139. doi: 10.1099/00207713-50-1-135/CITE/REFWORKS.

Frost, P. and Ness, A. (1997) 'Vaccination of Atlantic salmon with recombinant VP2 of infectious pancreatic necrosis virus (IPNV), added to a multivalent vaccine, suppresses viral replication following IPNV challenge', Fish & Shellfish Immunology. Academic Press, 7(8), pp. 609–619. doi: 10.1006/FSIM.1997.0113.

Galili, T. (2015) 'dendextend: an R package for visualizing, adjusting and comparing trees of hierarchical clustering', Bioinformatics. Oxford Academic, 31(22), pp. 3718–3720. doi: 10.1093/BIOINFORMATICS/BTV428.

Gismervik, K., Gåsnes, S., Gu, J., Stien, L., Madaro, A., Nilsson, J. (2019) 'Thermal injuries in Atlantic salmon in a pilot laboratory trial', Veterinary and Animal Science. Elsevier B.V., 8, p. 100081. doi: 10.1016/j.vas.2019.100081.

Graphpad (2007) 'Graphpad Prism for Windows'. San Diego California USA: Graphpad Software Inc.

Gudmundsdóttir, B. K. and Björnsdóttir, B. (2007) 'Vaccination against atypical furunculosis and winter ulcer disease of fish', Vaccine. Elsevier BV, 25(30), pp. 5512–5523. doi: 10.1016/j.vaccine.2007.02.009.

Gulla, S., Nilsen, H., Olsen, A., Colquhoun, D. (2020) 'Fiskepatogene *Pasteurella* i Norge', Norsk Fiskeoppdrett 11. Available at: https://www.vetinst.no/nyheter/fiskepatogene-pasteurella-i-norge (Accessed: 16 November 2021).

Hansen, M., Bojesen, A. and Planet, P. (2016) '*Pasteurella skyensis* strain 95A1 chromosome, complete genome - Nucleotide – NCBI', National Library of Medicine. Available at: https://www.ncbi.nlm.nih.gov/nuccore/CP016180 (Accessed: 2 May 2022).

Hordvik, I. (2015) 'Immunoglobulin isotypes in atlantic salmon, Salmo salar', Biomolecules. MDPI AG, 5(1), pp. 166–177. doi: 10.3390/BIOM5010166.

Jones, M. and Cox, D. (1999) 'Clinical disease in seafarmed Atlantic salmon (*Salmo salar*) associated with a member of the family Pasteurellaceae - a case history', Bulletin of the European Association of Fish Pathologists (United Kingdom). doi: 10.3/JQUERY-UI.JS.

Karlsson, M., Möllegård, I., Stiernstedt, G., Wretlind, B. (1989) 'Comparison of Western blot and enzyme-linked immunosorbent assay for diagnosis of Lyme borreliosis', European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. Eur J Clin Microbiol Infect Dis, 8(10), pp. 871–877. doi: 10.1007/BF01963773.

Kozlov, A., Darriba, D., Flouri, T., Morel, B., Stamatakis, A. (2019) 'RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference', Bioinformatics. Oxford Academic, 35(21), pp. 4453–4455. doi: 10.1093/BIOINFORMATICS/BTZ305.

Laemmli, U. K. (1970) 'Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4', Nature 1970 227:5259. Nature Publishing Group, 227(5259), pp. 680–685. doi: 10.1038/227680a0.

Legård, B. K. and Strøm, S. B. (2020) 'Pasteurellosis in Atlantic salmon (*Salmo salar*) in Western Norway', European Association of Fish Pathologist, 40(4), pp. 1–8. Available at: https://eafp.org/bulletin-archive/2020-volume40/issue_4/#wpfb-cat-267 (Accessed: 15 November 2021).

Legemiddelforskriften (2009) 'Forskrift om legemidler', FOR-2009-12-18-1839. Available at: https://lovdata.no/dokument/SF/forskrift/2009-12-18-1839.

Li, J., Barreda, D., Zhang, Y., Boshra, H., Gelman, A., LaPatra, S., Tort, L., Sunyer, J. (2006) 'B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities', Nature immunology, 7(10), pp. 1116–1124. doi: 10.1038/ni1389.

Litleskare, I. (2022) 'Bruk av legemidler i fiskeoppdrett', Norwegian Institute of Public Health. Available at: https://www.fhi.no/hn/legemiddelbruk/fisk/2021-bruk-av-legemidler-i-fiskeoppdrett/ (Accessed: 10 May 2022).

Mahanty, S., Prigent, A. and Garraud, O. (2015) 'Immunogenicity of infectious pathogens and vaccine antigens', BMC immunology. BioMed Central Ltd., 16(1), pp. 31–31. doi: 10.1186/s12865-015-0095-y.

Mashoof, S. and Criscitiello, M. F. (2016) 'Fish Immunoglobulins', Biology. Multidisciplinary Digital Publishing Institute (MDPI), 5(4). doi: 10.3390/BIOLOGY5040045.

Merino, S. and Tomás, J. M. (2015) 'Bacterial Capsules and Evasion of Immune Responses', eLS. Wiley, pp. 1–10. doi: 10.1002/9780470015902.A0000957.PUB4.

Microsoft Corporation (2022) 'Microsoft® Excel® for Microsoft 365 MSO'. Available at: https://office.microsoft.com/excel.

Mondal, H. and Thomas, J. (2022) 'A review on the recent advances and application of vaccines against fish pathogens in aquaculture', Aquaculture International 2022. Springer, pp. 1–30. doi: 10.1007/S10499-022-00884-W.

Morgulis, A., Coulouris, G., Raytselis, Y., Madden, T., Agarwala, R., Schäffer, A. (2008) 'Database indexing for production MegaBLAST searches', Bioinformatics (Oxford, England). Bioinformatics, 24(16), pp. 1757–1764. doi: 10.1093/BIOINFORMATICS/BTN322.

Murphy, K. and Weaver, C. (2017) 'Janeway's immunobiology', 9th edn. Edited by M. Toledo. New York and London: Garland Science, Taylor & Francis Group, LLC.

Nilsson, J., Moltumyr, L., Madaro, A., Kristiansen, T., Gåsnes, S., Mejdell, C., Gismervik, K., Stien, L. (2019) 'Sudden exposure to warm water causes instant behavioural responses indicative of nociception or pain in Atlantic salmon', Veterinary and Animal Science. Elsevier B.V., 8, p. 100076. doi: 10.1016/j.vas.2019.100076.

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., Prjibelski, A., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., Clingenpeel, S., Woyke, T., McLean, J., Lasken, R., Tesler, G., Alekseyev, M., Pevzner, P. (2013) 'Assembling single-cell genomes and mini-metagenomes from chimeric MDA products', Journal of computational biology : a journal of computational molecular cell biology. J Comput Biol, 20(10), pp. 714–737. doi: 10.1089/CMB.2013.0084.

Page, A., Cummins, C., Hunt, M., Wong, V., Reuter, S., Holden, Matthew T., Fookes, M., Falush, D., Keane, J., Parkhill, J. (2015) 'Roary: rapid large-scale prokaryote pan genome analysis', Bioinformatics. Oxford Academic, 31(22), pp. 3691–3693. doi: 10.1093/BIOINFORMATICS/BTV421.

R Core Team (2020) 'R: A Language and Environment for Statistical Computing'. Vienna, Austria: R Foundation for Statistical Computing. Available at: https://www.r-project.org/.

Ramírez-Paredes, J., Mendoza-Roldan, M., Lopez-Jimena, B., Shahin, K., Metselaar, M., Thompson, K., Penman, D., Richards, R., Adams, A. (2019) 'Whole cell inactivated autogenous vaccine effectively protects red Nile tilapia (*Oreochromis niloticus*) against francisellosis via intraperitoneal injection', Journal of Fish Diseases. John Wiley & Sons, Ltd, 42(8), pp. 1191–1200. doi: 10.1111/JFD.13041.

Reglinski, M., Lynskey, N., Choi, Y., Edwards, R., Sriskandan, S. (2016) 'Development of a multicomponent vaccine for Streptococcus pyogenes based on the antigenic targets of IVIG', Journal of Infection. W.B. Saunders, 72(4), pp. 450–459. doi: 10.1016/J.JINF.2016.02.002.

Rey, G. U. (2020) 'You Don't Need the Whole Antibody, Virology - About viruses and viral disease', Available at: https://www.virology.ws/2020/10/01/you-dont-need-the-whole-antibody/ (Accessed: 1 April 2022).

Ripley, B., Venables, B., Bates, D., Firth, D., Hornik, K., Gebhardt, A. (2002) 'Modern Applied Statistics with S. Fourth', Springer. Fourth. New York: Springer. Available at: http://www.stats.ox.ac.uk/pub/MASS4/ (Accessed: 4 May 2022).

Romstad, A., Reitan, L., Midtlyng, P., Gravningen, K., Evensen, Ø. (2012a) 'Development of an antibody ELISA for potency testing of furunculosis (*Aeromonas salmonicida* subsp *salmonicida*) vaccines in Atlantic salmon (*Salmo salar* L)', Biologicals, 40(1), pp. 67–71. doi: 10.1016/J.BIOLOGICALS.2011.09.011.

Romstad, A., Reitan, L., Midtlyng, P., Gravningen, K., Evensen, Ø. (2012b) 'Antibody responses correlate with antigen dose and in vivo protection for oil-adjuvanted, experimental furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*) vaccines in Atlantic salmon (*Salmo salar* L.) and can be used for batch potency testing of vaccines', Vaccine, 31(5), pp. 791–796. doi: 10.1016/j.vaccine.2012.11.069.

Rønneseth, A., Wergeland, H., Devik, M., Evensen, Ø., Pettersen, E. (2007) 'Mortality after IPNV challenge of Atlantic salmon (*Salmo salar* L.) differs based on developmental stage of fish or challenge route', Aquaculture, 271(1–4), pp. 100–111. doi: 10.1016/J.AQUACULTURE.2007.03.021.

Rønneseth, A., Ghebretnsae, D., Wergeland, H., Haugland, G. (2015) 'Functional characterization of IgM+ B cells and adaptive immunity in lumpfish (*Cyclopterus lumpus* L.)', Developmental and comparative immunology. Elsevier Ltd, 52(2), pp. 132–143. doi: 10.1016/j.dci.2015.05.010.

Saito, Y., Nishio, K., Yoshida, Y., Niki, E. (2005) 'Cytotoxic effect of formaldehyde with free radicals via increment of cellular reactive oxygen species', Toxicology. Elsevier, 210(2–3), pp. 235–245. doi: 10.1016/J.TOX.2005.02.006.

Sandlund, N., Rønneseth, A., Ellul, R., Nylund, S., Sandlund, L. (2021) '*Pasteurella* spp. Infections in Atlantic salmon and lumpsucker', Journal of fish diseases. John Wiley and Sons Inc, 44(8), pp. 1201–1214. doi: 10.1111/jfd.13381.

Seemann, T. (2014) 'Prokka: rapid prokaryotic genome annotation', Bioinformatics. Oxford Academic, 30(14), pp. 2068–2069. doi: 10.1093/BIOINFORMATICS/BTU153.

Skretting AS (2012) 'Fôr', Available at: https://www.skrettingguidelines.com/readimage.aspx?pubid=cd8a45bd-0e6e-409c-a2ee-1da2b7d19b06 (Accessed: 20 April 2022).

Soegiarto, G. (2021) 'Respons imun terhadap vaksin COVID-19 dan komorbid sebagai pertimbangan kehati-hatian', Available at: https://rsudrsoetomo.jatimprov.go.id/wp-content/uploads/2021/01/Respons-imun-terhadap-vaksin-COVID-19-dan-komorbid-sebagai-precaution-Gatot-Soegiarto-2021-PDF-1.pdf (Accessed: 1 April 2022).

Sommerset, I., Krossøy, B., Biering, E., Frost, P. (2005) 'Vaccines for fish in aquaculture', Expert review of vaccines. Taylor & Francis, 4(1), pp. 89–101. doi: 10.1586/14760584.4.1.89.

Sommerset, I., Bang, J., Bornø B., Haukaas A., og Brun E. (2021) 'Fiskehelserapporten 2020', Available at: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2021/fiskehelserapporten-2020 (Accessed: 15 November 2021).

Sommerset, I., Walde, C., Bang, J., Wiik-Nielsen, J., Bornø, G., Oliveira, V., Haukaas A., Brun E. (2022) 'Fiskehelserapporten 2021', Available at: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2022/fiskehelserapporten-2021.

Thorarinsson, R., Wolf, J., Inami, M., Phillips, L., Jones, G., Macdonald, A., Rodriguez, J., Sindre, H., Skjerve, E., Rimstad, E., Evensen, Ø. (2021) 'Effect of a novel DNA vaccine against pancreas disease caused by salmonid alphavirus subtype 3 in Atlantic salmon (*Salmo salar*)', Fish & Shellfish Immunology. Academic Press, 108, pp. 116–126. doi: 10.1016/J.FSI.2020.12.002.

Valheim, M., Håstein, T., Myhr, E., Speilberg, L., Ferguson, H. (2000) 'Varracalbmi: A new bacterial panophthalmitis in farmed Atlantic salmon, *Salmo salar* L.', Journal of Fish Diseases, 23(1), pp. 61–70. doi: 10.1046/J.1365-2761.2000.00209.X.

Vaxxinova (2021) 'Autogenous vaccines', Available at: https://en.vaxxinova.no/autogenous-vaccines/ (Accessed: 29 November 2021).

Vogel, F. R. and Sarver, N. (1995) 'Nucleic acid vaccines', Clinical Microbiology Reviews. American Society for Microbiology, 8(3), pp. 406–410. doi: 10.1128/cmr.8.3.406-410.1995.

Valheim, M., Håstein, T., Myhr, E., Speilberg, L., Ferguson, H. (2016) 'gplots: Various R programming tools for plotting data. CRAN. Available at: https://cran.r-project.org/web/packages/gplots/index.html (Accessed: 4 May 2022).

Wickham, H., Averick, M., Bryan, J., Yutani, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., Takahashi, K., Vaughan, D., Wilke, C., Woo, K., Yutani, H. (2019) 'Welcome to the {tidyverse}'. Journal of Open Source Software, p. 1686. doi: 10.21105.

Wickham, H. and Bryan, J. (2019) 'readxl: Read Excel Files'. Available at: https://cran.r-project.org/package=readxl.

Wizemann, T. M., Adamou, J. E. and Langermann, S. (1999) 'Adhesins as targets for vaccine development', Emerging infectious diseases. Centers for Disease Control and Prevention (CDC), 5(3), pp. 395–403. doi: 10.3201/eid0503.990310.

Wright, E. S. (2016) 'Using DECIPHER v2.0 to analyze big biological sequence data in R', R Journal. Technische Universitaet Wien, 8(1), pp. 352–359. doi: 10.32614/RJ-2016-025.

Zhang, Z., Schwartz, S., Wagner, L., Miller, W. (2000) 'A greedy algorithm for aligning DNA sequences', Journal of computational biology : a journal of computational molecular cell biology. J Comput Biol, 7(1–2), pp. 203–214. doi: 10.1089/10665270050081478.

# 7 – Appendix A

# Recipes for solutions used in the study

## 7.1 – Liquid growth media (TSB+)

Liquid growth media (TSB+) 1150g:

| | |
|---|---|
| Tropic soy broth (TSB) – BD – catalog# 211825 – lot# 1068445 | 30 g |
| NaCl – Honeywell International – catalog# 31434 – lot# L2180 | 15 g |
| Fetal calf serum (FCS) – Gibco – catalog# 10099-141 – lot# 2094466RP | 105 g |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 1000 g |

## 7.2 – NaOH 10M

NaOH 10M 100 ml:

| | |
|---|---|
| NaOH – Sigma Aldrich – catalog# S5881 – lot# 106K0004 | 4.00 g |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 100 ml total volume |

## 7.3 – PBS solution

PBS solution 1000 ml:

| | |
|---|---|
| $Na_2HPO_4$*$2H_2O$ – Sigma-Aldrich – catalog# 30435 – lot# SZBE0760V | 7.2 g |
| $KH_2PO_4$ – Sigma-Aldrich – catalog# 60220 – lot# SZBD1630V | 0.27 g |
| NaOH 10M – Appendix 7.2 | pH = 7.3 |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 1000 ml total volume |

## 7.4 – EDTA PBS solution

EDTA PBS solution:

| | |
|---|---|
| PBS solution – Appendix 7.3 | 990 ml |
| 0.5M EDTA – Life Technologies Corporation – catalog# 15575-038 – lot# 1920768 | 10 ml |
| NaOH 10M – Appendix 7.2 | pH = 7.28 |
| HCl 12M – Sigma-Aldrich – catalog# 258148 – lot# SZBF1100V | pH = 7.28 |

## 7.5 – PBS-Tween solution

PBS-Tween solution 1000.5 ml:

| | |
|---|---|
| PBS solution – Appendix 7.3 | 1000 ml |
| Tween - Sigma-Aldrich – catalog# P1379 – lot# SZBD2190V | 500 µl |

## 7.6 – PBS blocking solution

PBS blocking solution 103 ml:

| | |
|---|---|
| PBS solution – Appendix 7.3 | 100 ml |
| Skimmed milk powder – Sigma-Aldrich – catalog# 70166 – lot# BCBV5694 | 3.00 g |

## 7.7 – 0.1M Citric acid solution

Citric acid 1000 ml:

| | |
|---|---|
| Citric acid monohydrate – Sigma-Aldrich – catalog# 1909 – lot# SLBV1306 | 21.0 g |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 1000 ml total volume |

## 7.8 – 0.2M $Na_2HPO_4$*$2H_2O$ solution

0.2M $Na_2HPO_4$ 1000 ml:

| | |
|---|---|
| $Na_2HPO_4$ – Sigma-Aldrich – catalog# 30435 – lot# SZBE0760V | 35.6 g |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 1000 ml total volume |

## 7.9 – Phosphate-Citrate Buffer

Phosphate-Citrate Buffer 100 ml:

| | |
|---|---|
| 0.1M Citric acid solution – Appendix 7.7 | 24.3 ml |
| 0.2M $Na_2HPO_4$*$2H_2O$ solution – Appendix 7.8 | 25.7 ml |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 50 ml |

## 7.10 – Peroxidase substrate solution

Peroxidase substrate solution 37.5 ml:

| | |
|---|---|
| Phosphate-Citrate Buffer – Appendix 7.9 | 37.5 ml |
| o-Phenyleneidamine – Sigma-Aldrich – catalog# P4664 – lot# SLBT1774 | 15 mg |
| 30% $H_2O_2$ – Sigma-Aldrich – catalog# H1009 – Lot# S41966-307 | 15 µl |

## 7.11 – 2.5M $H_2SO_4$ solution

2.5M $H_2SO_4$ solution 100 ml:

| | |
|---|---|
| $H_2SO_4$ – Professional Clinical Laboratory Inc. – catalog# 20.690.293 – lot# NO23 | 6.94 ml |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 93.06 ml |

## 7.12 – 50x TAE buffer

50x TAE buffer 1000 ml:

| | |
|---|---|
| Tris Base – Sigma-Aldrich – catalog# T1503 – lot# SLBV1855 | 242 g |
| 0.5M EDTA – Life Technologies Corporation – catalog# 15575-038 – lot# 1920768 | 100 ml |
| Acetic acid – Honeywell International – catalog# 33209 – lot# SZBF3510 | 57.1 ml |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 1000 ml total volume |

## 7.13 – 1x TAE buffer

1x TAE buffer 1000 ml:

| | |
|---|---|
| 50x TAE buffer – Appendix 7.12 | 20 ml |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 980 ml |

## 7.14 – 0.5M Tris-HCl

0.5M Tris-HCl 1000 ml:

| | |
|---|---|
| Tris Base – Sigma-Aldrich – catalog# T1503 – lot# SLBV1855 | 6 g |
| HCl 12M – Sigma-Aldrich – catalog# 258148 – lot# SZBF1100V | pH = 6.8 |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 100 ml total volume |

## 7.15 – Sample buffer

Sample buffer 9.5 ml:

| | |
|---|---|
| 0.5M Tris-HCl – Appendix 7.14 | 1.25 ml |
| Glycerol – Sigma-Aldrich – catalog# G5516-1L – lot# SHBG0744V | 2.5 ml |
| Sodium dodecyl sulfate – Sigma Aldrich – catalog# L4390 – lot# SLBB0911V | 200 mg |
| 0.5% Bromophenol blue – Merck – catalog# 8122.0025 – lot# L631122 | 200 µl |
| β-mercaptoethanol – Sigma Aldrich – catalog# M3148 – lot# SHBG9616V | 500 µl |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 3.55 ml |

## 7.16 – Fixative solution

Fixative solution 160 ml:

| | |
|---|---|
| Methanol – Honeywell International – catalog# 32213 – lot# SZBG2740H | 100 ml |
| Acetic acid – Honeywell International – catalog# 33209 – lot# SZBF3510 | 20 ml |
| Fixative Enhancer Concentrate – Bio-Rad – catalog# 161-0461 – lot# 64075750 | 20 ml |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 20 ml |

## 7.17 – Silver stain solution

Silver stain solution 65 ml:

| | |
|---|---|
| Silver Complex Solution – Bio-Rad – catalog# 161-0462 – lot# 64021566 | 5 ml |
| Reduction Moderator Solution – Bio-Rad – catalog# 161-0463 – lot# 210012137 | 5 ml |
| Image Development Reagent – Bio-Rad – catalog# 161-0464 – lot# P50762 | 5 ml |
| Development Accelerator Solution – Bio-Rad – catalog# 161-0449 – lot# 64107923 | 50 ml |

## 7.18 – Blotting buffer

Blotting buffer 1000 ml:

| | |
|---|---|
| Tris Base – Sigma-Aldrich – catalog# T1503 – lot# SLBV1855 | 3.028 g |
| Glycine – Sigma-Aldrich – catalog# G7126 – lot# BCBJ3736V | 14.4g |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 800 ml total volume |
| Methanol – Honeywell International – catalog# 32213 – lot# SZBG2740H | 200 ml |

## 7.19 – TBS solution

TBS solution 500 ml:

| | |
|---|---|
| NaCl – Honeywell International – catalog# 31434 – lot# L2180 | 14.61 g |
| Tris Base – Sigma-Aldrich – catalog# T1503 – lot# SLBV1855 | 1.21 g |
| HCl 12M – Sigma-Aldrich – catalog# 258148 – lot# SZBF1100V | pH = 7.5 |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 500 ml total volume |

## 7.20 – TBS blocking solution

TBS blocking solution 105 ml:

| | |
|---|---|
| Skimmed milk powder – Sigma-Aldrich – catalog# 70166 – lot# BCBV5694 | 5 g |
| TBS solution – Appendix 7.19 | 100 ml |

## 7.21 – TTBS

TTBS 700 ml:

| | |
|---|---|
| TBS solution – Appendix 7.19 | 700 ml |
| Tween - Sigma-Aldrich – catalog# P1379 – lot# SZBD2190V | 350 µl |

## 7.22 – Antibody buffer

Antibody buffer 202 ml:

| | |
|---|---|
| Skimmed milk powder – Sigma-Aldrich – catalog# 70166 – lot# BCBV5694 | 2 g |
| TTBS – Appendix 7.21 | 200 ml |

## 7.23 – 5% acetic acid solution

5% acetic acid solution 1000 ml:

| | |
|---|---|
| Acetic acid – Honeywell International – catalog# 33209 – lot# SZBF3510 | 5 ml |
| Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System | 995 ml |

# 8 – Appendix B – Overview of ELISA assays, statistics, and molecular weight in SDS-PAGE gels.

Table 7 – Overview of antigen, antisera, and dilution of antisera used for all ELISA assays.

| Assay | Antigen | Antisera | Dilution of antisera |
|---|---|---|---|
| 1 | PaL-1 | PaL-1 Fish 1-10 | 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600 |
| 2 | PaL-2 | PaL-2 Fish 1-10 | 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600 |
| 3 | PaL-3 | PaL-3 Fish 1-11 | 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600 |
| 4 | PaL-1 | Control- Fish 1-10 | 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600 |
| | PaL-2 | | |
| | PaL-3 | | |
| 5 | PaL-1 | PaL-2 Fish 1-10<br>PaL-3 Fish 1-11 | 1:400 |
| | PaL-2 | PaL-1 Fish 1-10<br>PaL-3 Fish 1-11 | |
| | PaL-3 | PaL-1 Fish 1-10<br>PaL-2 Fish 1-10 | |
| 6 | PaL-1 | PaL-1Fish 1-10<br>Control- fish 1-10 | 1:400 |
| | PaL-2 | PaL-2 Fish 1-10<br>Control- fish 1-10 | |
| | PaL-3 | PaL-3 Fish 1-11<br>Control- fish 1-10 | |
| 7 | PaL-1 | PaL-1 Fish 1-10<br>PaL-2 Fish 1-10<br>PaL-3 Fish 1-11<br>Control- fish 1-10 | 1:400 |
| | PaL-2 | | |
| | PaL-3 | | |
| 8 | RK-1 | PaL-1 Fish 1-10<br>PaL-2 Fish 1-10<br>PaL-3 Fish 1-11<br>Control- fish 1-10<br>Control+ fish 1-10 | 1:400 |

Table 8 – Overview of all Mann–Whitney U test of the ELISA assays. Significant differences were based on p-values and considered significant if $p<0.05$.

| Test | Coating antigen | p-value | P value summary | Significant differences (yes/no) |
|---|---|---|---|---|
| PaL-1 vs Control- | PaL-1 | p<0.0001 | *** | yes |
| PaL-2 vs Control- | PaL-1 | p<0.0001 | *** | yes |
| PaL-3 vs Control- | PaL-1 | 0.0001 | *** | yes |
| PaL-1 vs PaL-2 | PaL-1 | 0.0630 | - | no |
| PaL-1 vs PaL-3 | PaL-1 | 0.1697 | - | no |
| PaL-2 vs PaL-3 | PaL-1 | 0.8603 | - | no |
| PaL-1 vs Control- | PaL-2 | p<0.0001 | *** | yes |
| PaL-2 vs Control- | PaL-2 | p<0.0001 | *** | yes |
| PaL-3 vs Control- | PaL-2 | 0.0001 | *** | yes |
| PaL-1 vs PaL-2 | PaL-2 | 0.3930 | - | no |
| PaL-1 vs PaL-3 | PaL-2 | 0.5974 | - | no |
| PaL-2 vs PaL-3 | PaL-2 | 0.0726 | - | no |
| PaL-1 vs Control- | PaL-3 | p<0.0001 | *** | yes |
| PaL-2 vs Control- | PaL-3 | p<0.0001 | *** | yes |
| PaL-3 vs Control- | PaL-3 | 0.0001 | *** | yes |
| PaL-1 vs PaL-2 | PaL-3 | 0.1655 | - | no |
| PaL-1 vs PaL-3 | PaL-3 | 0.4181 | - | no |
| PaL-2 vs PaL-3 | PaL-3 | 0.6985 | - | no |
| PaL-1 vs Control- | RK-1 | 0.0630 | - | no |
| PaL-2 vs Control- | RK-1 | 0.4813 | - | no |
| PaL-3 vs Control- | RK-1 | 0.0005 | *** | yes |
| PaL-1 vs Control+ | RK-1 | p<0.0001 | *** | yes |
| PaL-2 vs Control+ | RK-1 | p<0.0001 | *** | yes |
| PaL-3 vs Control+ | RK-1 | p<0.0001 | *** | yes |
| PaL-1 vs PaL-2 | RK-1 | 0.3527 | *** | yes |
| PaL-1 vs PaL-3 | RK-1 | 0.0221 | *** | yes |
| PaL-2 vs PaL-3 | RK-1 | 0.0017 | *** | yes |
| Control- vs Control+ | RK-1 | p<0.0001 | *** | yes |

Table 9 – Overview of Mann–Whitney U test of weight gain throughout the vaccination assays. Significant differences were based on p-values and considered significant if $p<0.05$.

| Test | p-value | P value summary | Significant differences (yes/no) |
|---|---|---|---|
| PaL-1 vs CTRL | 0.5452 | - | No |
| PaL-2 vs CTRL | 0.7054 | - | No |
| PaL-3 vs CTRL | 0.0124 | * | Yes |
| PaL-1 vs PaL-2 | 0.6842 | - | No |
| PaL-1 vs PaL-3 | 0.0317 | * | Yes |
| PaL-2 vs PaL-3 | 0.0183 | * | Yes |

Table 10 – Overview of the approximate molecular weight of the protein bands of the silver stained SDS-PAGE gels, as well as the annotation to each band, it's closest similar band, and the difference to the closest similar band. Molecular weight is measured with a ruler and the ladder as reference.

| Genomovar | Band | Approximate molecular weight (kDa) | Closest similar band | Difference to closest similar band (kDa) |
|---|---|---|---|---|
| Genomovar *cyclopteri* | A | 99 | J | 31 |
| Genomovar *cyclopteri* | B | 55 | C | 9 |
| Genomovar *cyclopteri* | C | 46 | B | 9 |
| Genomovar *cyclopteri* | D | 36 | K | 2 |
| Genomovar *cyclopteri* | E | 32 | K and L | 2 |
| Genomovar *cyclopteri* | F | 25 | M | 1 |
| Genomovar *cyclopteri* | G | 22 | M | 2 |
| Genomovar *cyclopteri* | H | 19 | G | 3 |
| Genomovar *salmonicida* | I | 131 | A | 32 |
| Genomovar *salmonicida* | J | 68 | B | 13 |
| Genomovar *salmonicida* | K | 34 | D and E | 2 |
| Genomovar *salmonicida* | L | 30 | E | 2 |
| Genomovar *salmonicida* | M | 24 | F | 1 |