Intracellular bacterial pathogens in aquaculture: vaccines and virulence factors

Analysis of membrane vesicle based vaccine against Piscirickettsia salmonis and evaluation of type IV pili system presence in Francisella noatunensis subsp. noatunensis

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Abstract

Fish farming is the most common form of aquaculture. Its production contributes close to 50% of total global fish production, and is estimated to increase to 62% by year 2030. Piscirickettsiosis and francisellosis are two common diseases in Atlantic salmon and cod respectively. These diseases arise due to the infection of pathogenic bacteria *Piscirickettsia salmonis* (piscirickettsiosis) and *Francisella noatunensis* subspecies *noatunensis* (francisellosis). Due to the absence of vaccines with a sufficiently long-lasting effect, the current strategy for both protection and treatment of these common aquaculture diseases is through the use of antibiotics. Unnecessary use of antibiotics is a global problem, and is an important factor in the emergence of multi-resistant bacterial strains. In this thesis, the focus has been on examining the effect from a vaccine trial against piscirickettsiosis in Atlantic salmon, using membrane vesicles isolated from *P. salmonis,* as well as evaluating the presence of a type IV pili system associated with virulence in *F.n.n*.

Cumulative mortality obtained from the vaccine trial, indicates that no protective effect were induced by immunization with MVs. The reason for this was that no difference in onset of death nor total cumulative mortality were seen between control and vaccine groups, under the conditions tested. However, a potentially positive effect was detected for the immunized groups, as gene expression of both Interleukin-12 and macrophage-expressed gene were statistically significantly increased at 4 weeks post challenge. Histological analysis comparing tissue from immunized fish before and after challenge with *P. salmonis* did not show any apparent granulomas, otherwise commonly seen in fish infected with this pathogen. However, several other indications of disease could be seen in both spleen and kidney of infected fish, and could be related to the infection of *P. salmonis*, due to their presence only post infection. No granulomas were detected in neither control nor immunized group, and no defined differences in disease progression were seen between the groups.

The presence of a type IV pili system was evaluated in *F.n.n.* by utilizing the methods immunogold labeling, atomic force microscopy, and flow cytometry analysis. *F.n.n.* mutants containing His-tagged pili proteins were used as markers for presence of pili, by the use of anti-His antibodies. Mutants containing a disrupted type IV pili component PilD along with His-tagged pilins, as well as mutants with only His-tagged pilins were examined. Disruption of the PilD pili subcomponent were investigated, to assess any difference in expression of pili. No pili

nor type IV pili-like appendages were discovered using any of these techniques, on any of the mutants. However, heavy gold labeling was seen on the surface of several *F.n.n.* Bacteria positively labeled with the anti-His antibody were sorted using a flow cytometry, but neither an increase in gold labeling nor the presence of pili were seen. Because heavy labeling was present mainly in damaged or lysed bacteria, it is uncertain if this labeling is due to attachment to leaked internal components or if the condition itself leads to non-specific binding of antibodies.

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Abbreviations

AFM	Atomic force microscope
AID	Activation-induced cytidine deaminase
AMP	Antimicrobial peptide
APC	Antigen presenting cell
BCR	B-cell receptor
BSA	Bovine serum albumine
Ct	Cycle threshold
DPC	Days post challenge
EB	Eugon broth
ECA	Eugon chocolate agar
E. coli	*Escherichia coli*
FSC	Forward scatter
Ef1α	Elongation factor 1 α
EV	Extracellular vesicles
FAO	Food and agriculture organization of the united nations
F.n.n.	*Francisella noatunensis* subsp. *noatunensis*
F.n.o	*Francsiella noatunensis* subsp. *orientalis*
F.t.t.	*Francisella tularensis* subsp. *tularensis*
FPR	*Francisella* pathogenicity Island
FSG	Fish skin gelatin
Fw	Forward
GC	Germinal centers
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISA	Infectious salmon anemia

Kan	Kanamycin
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACPF	Membrane attack complex/perforin
MBL	Mannose binding lectin
MHC	Major Histocompatibility complex
MPEG	Macrophage-expressed gene
MIC	Minimal inhibitory concentration
MMC	Melanomacrophage centers
MV	Membrane vesicles
MVE	Multivesicular endosome
NEB	New England biolabs
NK	Natural killer (cell)
NLR	NOD-like receptors
MV	Membrane vesicles
PAG	Protein-A gold
PAMP	Pathogen associated molecular patterns
PAS	Periodic acid-Schiff (stain)
PCR	Polymerase chain reaction
PI	Propidium iodide
P. salmonis	*Piscirickettsia salmonis*
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
Rv	Reverse
SOCS	Suppressor of cytokine signaling
Subsp.	Subspecies

SRS	Salmonid rickettsial septicemia
SSC	Side scatter
TCR	T-cell receptor
TEM	Transmission electron microscope
Tfp	Type IV pili/pili
Th	T helper cell
TLR	Toll-like receptors
T4SS	Type IV secretion system
UA	Uranyl Acetate
WHO	World Health Organization
WPC	Weeks post challenge

1 Introduction

Aquaculture is the process of breeding, maintaining, and harvesting of aquatic organisms, often in optimal artificial environments or net-pens. This form of farming includes fish, mollusks, algae etc. with fish farming being the most common and fastest growing industry globally. With a growing human population, the need for a reliable food source is becoming increasingly important. According to the Food and Agriculture Organization (FAO), aquaculture represents close to 50% of total global fish production. Although salmon is valued for both its food and fat sources, the major species produced in world aquaculture are carp (grass, sliver and common carp) which contributed to 29% of total production in 2016 vs. 4% for Atlantic salmon (*salmo salar*) (1). According to the World Bank report from 2013, it is predicted that aquaculture will contribute to 62% of total food fish by year 2030 (2).

1.1.1 Salmon aquaculture

Atlantic salmon (*Salmo salar*) is by far the most abundant species of the farmed salmonids. Norway, the United Kingdom and Chile are the top three producers of Atlantic salmon globally (3). These salmonids spend their early life and juvenile stage in freshwater. When approaching adolescence, most Atlantic salmon will go through a process called smolting, which allows them to transfer from freshwater habitats to seawater. This process includes external and behavioral changes as well as increased salinity tolerance and growth rate (4). Mimicking a natural smolting process is hard to replicate in commercial hatcheries. Therefore, the observed survival rate of hatchery-reared smolts may be lower than that of wild fish when released into a natural environment. The probable reason for this is that fish from hatcheries are protected against some of the challenges and process of natural selections common in a natural environment (4). Even though salmon is not the major aquaculture species world-wide, its market value is not negligible and demand shows no signs of weakening (3).

1.1.2 Cod aquaculture

Atlantic cod (*Gadus morhua*) are a group of demersal fish (fish that lives on or near the bottom of the sea/lake). This group of fish has played an important part in commercial fishing along the coast of Norway as well as in Iceland and the United Kingdom (5). However, the amount wild cod populations is constantly fluctuating due to harvesting and, climate change, as well as competition between cohorts (group of fish born the same year within a population) (6). The Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima) has since 2003 been working on breeding and farming cod. The aim of their project is to breed cod with both better growth quality as well as being more resistant to fish diseases (7). The project showed promising results and reached a total of 15 cod hatcheries around Norway, however, commercial farming has since decreased due to high production cost, as well as problems with early sexual maturation and disease outbreaks (5). Nonetheless, work was still continued in optimizing cod breeding. According to Øyvind J. Hansen (Nofima), the recent fifth generation of farmed cod shows great characteristics of improved survival and growth rates, indicating a possible bright future for cod aquaculture (8).

1.2 Bacterial diseases in aquaculture

Much like humans, fish are also prone to contract diseases through viruses, parasites, bacteria etc. with the farming environments being especially vulnerable due to dense communities in enclosed areas. Diseases in aquaculture, constitute alone the largest single cause of economic losses, in which bacterial infections represents a major part of the problem (9). Bacteria are an abundant, diverse group of microorganisms capable of inhabiting all known habitats on earth. Their high division and mutation rate as well as genetic transformation makes bacteria well suited to overcome obstacles and securing the survival of the species. For this reason, new pathogenic bacterial species will continue to emerge as well as the ones already present may be able to further adapt and improve its pathogenicity (10). Both Gram-negative and -positive species are able to infect fish, though Gram-negative bacteria makes up the majority of the infections (11). The difference between Gram- negative and –positive bacteria is the structure of their outer membrane. The Gram-positive bacterial cell wall consist of the cytoplasmic membrane and a thick layer of peptidoglycan, which consist of a polysaccharide chain, linked with amino-acid peptides. The cell wall of Gram-negatives also contains a cytoplasmic membrane, but in contrast to the Gram-positives, the layer of peptidoglycan is much smaller.

In addition, Gram-negatives have an outer membrane, a second lipid bilayer (12) with a lipopolysaccharide (LPS) outer-layer. Two Gram-negative bacterial infectious diseases common among fish are piscirickettsiosis and francisellosis, and are caused by the bacterial pathogens *Piscirickettsia slamonis* and *Francisella noatunensis* respectively. These diseases are common among several fish used in aquaculture. Piscirickettsiosis is a widespread problem among Atlantic salmon, especially in Chile (13), while francisellosis is known to infect species such as Atlantic cod and tilapia (14). Bacterial infections are often diagnosed based on isolation/detection of the invading pathogen as well as clinical and behavioral symptoms in the infected fish.

1.3 Preventative measures and treatment

As aquaculture is an industry that is continuously growing, regimes for preventative measures are important to ensure a sustainable growth and maintenance. A study performed by Pulkkinen et. al showed that these environments not only were vulnerable areas for disease contraction. When studying the Gram-negative bacteria *Flavobacterium columnare* in farmed salmon, it was discovered that both the severity of disease symptoms and virulence of *F. columnare* had increased over 23 years. This finding suggested that fish-farming may be important sites for selective growth of virulent pathogens (15). Three factors are important for disease outbreaks, the microorganism, the fish itself and the environment. Environmental factors, such as temperature is especially important in disease formation. However, this factor is hard to influence (16). Reducing and preventing the outbreak of opportunistic bacteria can be done by administering antibiotics prophylactically, however this is unquestionably not sustainable, due to the increase in antimicrobial resistance. Instead, the single most important preventative measure for disease outbreaks, are the use of vaccines (17).

1.3.1 Antibiotics

A common strategy used for both prophylaxis and treatment of several diseases in aquaculture is the use of antibiotics. Antibiotics are usually administered through feed, immersion therapy (bath, dip, etc.) or injection. Medicated feed is advantageous in that it does not stress the fish, unlike immersion therapy and injection. However, uneaten as well as excreted antibiotics may enter the surrounding environment, which leads to accumulation of antibiotics in marine sediments, and can persist for months. In addition, a common behavioral disease symptom in

sick fish is loss of appetite, in which the oral route becomes unavailable (18, 19). Even though antibiotics are capable of disease prevention to a certain extent, its negative sides and disadvantages are much greater. It is well known that the use of antibiotics is linked to the emergence of multi-resistant bacteria, and it has therefore never been more important to reduce and control its usage. However, though the use of antibiotics has dramatically reduced over the last decades in countries such as Norway (17), other countries such as Chile are still struggling. The high use of antibiotics has caused a damaging image of Chilean salmon, especially within the US (3). Stricter regulations and new practices have come about, however, the lowest use of antibiotics in Chile was seen in 2010, and has since then fluctuated, signaling that a steady decrease has not been accomplished (20). The development of efficient vaccines is therefore crucial.

1.3.2 Vaccines in aquaculture

Vaccines has become an important part of a sustainable aquaculture and plays a vital role in the protection against pathogens. Not only are they able to prevent and induce long-time protection through activation of the adaptive immune system, but has also lowered the use and need for antibiotics. Vaccines in aquaculture are primarily used for prevention and control of infectious diseases. The use of vaccines has led to a reduced risk of disease development, as well as creating herd immunity in which non-vaccinated fish are protected against disease through the vaccinated fish (17, 21). The three common ways of vaccine administration is through feed, immersion therapy or injection. The three vaccine technologies most frequently used in aquaculture are inactivated, live attenuated and DNA vaccines.

Inactivated vaccines

Inactivated or non-replicating vaccines contains whole, killed pathogens (often referred to as bacterin) or immunogenic subunits. An advantage with this technology is the ability to induce an immune response without causing disease. Inactivation is carried out either by physical (UV light, heat etc.) or chemical (formaldehyde, detergents etc.) inactivation. However, this vaccine technology mainly provokes a B-cell response, which consist of circulating antibodies, that is less effective against intracellular pathogens (22).

Live attenuated vaccines

Live attenuated or replicating vaccines contains pathogens with reduced virulence, which are still able to survive and replicate. Several attenuation strategies may be employed. Laboratory passage involves passage of the pathogen though tissue culture and/or media, making it less virulent. Another way is by altering the genetic composition of the pathogen by creating random mutation(s) by physically or chemically induced mutagenesis, or by genetically modifying and disrupting virulence gene(s) or important metabolic pathway(s). As live attenuated pathogens are able to persist within its host, this vaccine technology is capable of inducing both cellular and humoral adaptive immune response. Activation of the cellular immune response (T-cells) is especially important when considering vaccine development against intracellular pathogens (23, 24).

DNA vaccines

Vaccines used in aquaculture most frequently consist of bacterin (inactivated vaccine) and adjuvant formulations. However, this method is less efficient for both intracellular bacterial and viral infections. DNA vaccines consist of genes encoding protective antigens, derived from the pathogen and inserted into bacterial plasmids. The plasmid is delivered by intramuscular injection and transfects (deliberate introduction) antigen presenting cells (APC). APC in turn, express the proteins introduced from the plasmid and presents these on major histocompatibility molecules (MHC). This method resembles a viral infection and activates both a cellular and humoral adaptive immune response (25).

Adjuvants

An adjuvant is added to vaccines to aid in and enhance immune response elicited by the vaccine. Two major roles of adjuvants is to act as a delivery system of the active vaccine component and to enhance the innate immune response (26). Delivery functions includes both protection and transfer, as well creating a depot effect of the active vaccine component. Common adjuvants are mineral and vegetable oils, but though they improve vaccine efficacy, they can only be administered by injection and are known to cause side effects. (27). For oil-adjuvants, a common side effect include intra-abdominal lesions, and mineral oil-adjuvants are linked to formation of granulomas at both injection site and in several organs (28, 29).

1.4 The adaptive and innate immune system

1.4.1 Atlantic salmon

Atlantic salmon is part of the teleost, which is a large class of ray-finned fishes, including the model organism zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). Like other vertebrates, the teleost has also shown a presence of an innate and adaptive immune system.

The innate immune system is a non-specific, first line defense mechanism common among vertebrates as well as invertebrates. The system can be divided into humoral and cellular units, which in turn can be divided into sensing and effector components. The humoral sensing components includes pattern recognition receptors (PRRs), such as pentraxins and mannose binding lectin (MBL). Examples of effector components are cytokines, lysosomes, and acute phase reactants (47). Cellular innate immune components include sensing units as toll-like receptors (TLRs) and NOD-like receptors (NLRs) (30). The effectors of the cellular immune components include antimicrobial peptides, proteases, lipases etc. Neutrophils and macrophages are important fish inflammatory cells, especially since fish do not possess lymph nodes. These phagocytic cells partake in the elimination of invading microorganisms by engulfing and internal destruction of the pathogens by antimicrobial peptides (AMPs), enzymes etc. (31).

The adaptive immune system is highly specific, although slower acting when compared to the innate. Unlike the innate, the goal of the adaptive system is to achieve long-term protection. The cells responsible for this immune reaction are mainly B- and T cells. Fish do not have bone marrow, and it is believed that B- and T-cells originate from the head kidney, an organ analogous to the mammalian adrenal gland (32, 33). B-cells are responsible for the secretion of soluble immunoglobulins (Ig, also known as antibodies) and may contain membrane-bound Ig (B-cell receptors/BCR). Igs possess specific antigen recognition binding sites, which binds to specific antigens and stimulates further engagement of the immune system, including T-cell activation (34). When soluble Igs binds to its target, it can elicit several functions including induction of phagocytosis, neutralization, as well as activation of the complement cascade. A naïve B-cell (unstimulated) can either become short-lived plasma cells (do not have BCR, only secretes Ig) or long-lasting memory cells. T-cell receptors (TCRs) are on the other hand always membrane-bound. There is little knowledge about the function of teleost T-cells. However, both cytotoxic and helper T-cells have been demonstrated in several fish species (35, 36).

Though several similar functions have been characterized between the teleost and mammalian adaptive system, some differences are present. In teleost, only three immunoglobulin isotype (IgM, IgD and IgZ/T) are characterized so far (37). The IgM response has also been observed to be slower, with the specific antibody titers after immunization being detected as late as 3-4 weeks after immunization (32, 34, 38).

As the innate immune response is evolutionary older than the adaptive, it may not be entirely inconceivable that fish, as an old, well-functioning species, rely more heavily on the innate immune system. This is further strengthened by the fact that multiple forms of C3 has been identified in the teleost, an important convertase of the complement system (34). The complement system is part of innate immunity and several homologs of mammalian components has been found in teleost. This system is comprised of three different pathways (classical, alternative and lectin pathway), in which the end goal of all pathways is the activation of C3, which in turn is part of several separate cascades leading to phagocytosis of pathogens as well as cell lysis and B-cell activation (39, 40). In addition, the fact that fish TLRs not only shows a greater diversity compared to mammalian counterparts, but also an additional six TLRs have been identified, indicates that though the adaptive immune system is less complex compared to mammals, a more advanced innate system may compensate for this (39).

Head kidney

The head kidney (upper part of kidney that is situated on the dorsal side, parallel to the vertebral column) is an immunologically active endocrine organ, which releases several hormones, among them cortisol and thyroid hormones (33). It plays an important role for immune-endocrine interactions and key regulatory functions, and can be compared to mammalian adrenal glands (37). The head kidney is also associated with hemopoietic function. In addition, it is one of the primary sites of leukocyte production as well as an important site for B cell proliferation, as teleost lack germinal centers (GC, mammalian site for B cell proliferation and differentiation)(34). As the head kidney plays an important role as both an endocrine and an immunologically active organ, bidirectional signaling between these areas of function is possible (33). Fish do not possess lymph nodes, and as such, the head kidney is considered the major lymphoid organ. Among other functions, it is important for the removal and trapping of debris and damaged cells from the blood stream, as well as antigen trapping (41).

Spleen

The spleen of teleost is also considered to partly function as a lymphoid organ, though poorly developed (41, 42). The organ is believed to mainly be involved in blood cell formation, storage, and filtering, as well as immune reactivity. While spleen of most mammalians are distinctly organized into red and white pulp (responsible for mechanical filtration of blood cells and lymph function, respectively), the spleen of fish does not possess this specific compartmentalization (43, 44). The spleen of fish contains ellipsoids, which are specialized capillary walls that contains lymphocytes and macrophages. The macrophages are commonly arranged in melanomacrophage centers, which are believed to carry out several important functions.

Melanomacrophage centers

Melanomacrophage centers (MMCs) are accumulations of pigmented phagocytes found in both spleen and head kidney, which shows structural similarities to mammalian germinal centers, but its function is not fully identified in teleost. These aggregates are distinguishable under a light microscope due to presence of a variety of pigments such as melanin, lipofuscin and hemosiderin (45, 46). Among salmonids, these centers are randomly dispersed in the lymphoid tissue, unlike other teleost where they form compact centers (47). The MMCs are suggested to possess important functions, both immunological and non-immunological. Functions that have been suggested for the MMCs are phagocytosis, storage of iron and unmetabolized materials, as well as B cell proliferation and differentiation (suggested due to the presence of activation-induced cytidine deaminase, AID, a mammalian enzyme in GCs important for B-cell Ig mutation (48)). The ability to phagocytose is not limited to only engulf erythrocytes (responsible for the pigmentation by hemosiderin and iron-storage) but also infectious material as pathogens, especially blood borne, as MMCs are closely associated with ellipsoids (45, 47, 49). Ellipsoids, also referred to as Schweigger-Seidel sheath, are specialized capillaries or arterioles in the spleen surrounded by macrophages (46, 50). A progressively increasing accumulation of iron is seen in spleen MMCs during hemolytic disease, suggesting its importance in iron-storage during breakdown of erythrocytes. This also highlights the difference these centers play in the lymphoid tissues, as this was not observed in the head-kidney MMCs (46, 47). In addition, carp (*Cyprinus carpio*) MMCs was shown to possess the ability of long-time antigen storage, indicating an important function in immunity acquisition (51).

Liver

The liver of fish is functionally comparable to the liver of mammals. This vital organ is responsible for detoxification, production of biochemicals that aids digestion, storage of carbohydrates and fats, etc. Like the spleen, the liver is also important for the destruction of old blood cells. Other important functions include nitrogen excretion and ensuring proper maintenance of blood chemistry.

Suppressor of cytokine signaling 3-like

SOCS are key negative regulators of growth factor and cytokine signaling. Several SOCS3 have been found in zebrafish, tilapia and rainbow trout (52). In humans, SOCS3 contains a motif (KIR) which functions as a kinase inhibitor of the janus kinase (tyrosine kinase important in the JAK-signal transducer and activator of transcription (STAT) signal transduction pathway)(53). JAK-STATs plays an important role in control of immune response as well as proliferation, apoptosis and oncogenesis (54, 55).

Macrophage-expressed gene 1

Macrophage-expressed gene 1 (*mpeg1*) is a gene that codes for a membrane attack complex/perforin (MACPF) protein perforin-2, which functions as part of the innate immune system (56). This protein has been highly evolutionary conserved and is present in both humans and teleost (57). Perforin-2 is situated in the membrane of several cell types, including phagocytes and B-cells (58) and it is unique, as it contains a MACPF domain on both the extracellular and cytosolic space (57, 59). Unlike other perforins, perforin-2 is therefore able to combat bacteria intracellularly. It has been shown to both be able to eliminate Gram-negative and –positive bacteria intracellularly. The current hypothesis is that perforin-2 containing vesicles fuses with the intracellular bacteria, and creates pores which allows entrance of AMPs as well as lysis of the cell (60). Unlike other perforins, perforin-2 secretion is not known, and its way of extracellular destruction of bacteria is still to be determined.

Major histocompatibility complex class II beta

Though little is known about APCs in fish, MHC class II are shown in some species to be expressed by mononuclear phagocytes (32, 61). In humans, MHC class II are expressed in the outer membrane of professional antigen-presenting cells (APCs: B-cells, dendritic cells, mononuclear phagocytes etc.). APC engulfs an antigen which is then digested into fragments (epitope) and extracellular represented on MHC II proteins. The MHC-II, epitope complex can then bind to a CD4+ helper T-cell and stimulates their proliferation, which in turn stimulates production of Igs and interferon γ (IFN-γ) activation of macrophages (62, 63).

Cytokines

Cytokines are proteins important for signaling and plays an essential role in initiation of the immune response, as well as maintenance and amplification. These soluble immune effectors functions by regulating cells by paracrine (between cells), autocrine (to itself) and endocrine (on distant cells) signaling. Though the characterization of fish cytokines is still in progress, several are identified and share functional similarities to human homologs (44, 64). Cytokines are a loose term and includes several groups of proteins such as interleukins, chemokines, and interferons. In this study, the interleukins IL-1β, -8, -10 and -12 were used for analyzing the immunological response after a vaccine trial. While IL-1β, IL-8 and IL-12 are pro-inflammatory interleukins, IL-10 is anti-inflammatory and acts as suppressors of the immune response. Pro-inflammatory cytokines are known for their systemic effects as neutrophil mobilization and elevation of body temperature (65).

Interleukin-1β

IL-1β is produced by several cell types, including macrophages and neutrophilic granulocytes. This cytokine is secreted in response to a host pattern recognition receptor (PRR) being activated, and is an important pro-inflammatory mediator during an infection (66, 67). IL-1β binds to its receptors, activating a signaling cascade which to leads to enhancement of immunological components. This cytokine acts as a chemoattractant for leukocytes, which is part of the early innate immune defense, as well as amplifying production of chemokines in cells present at an infection site (67, 68).

Interleukin 8

IL-8, also known as cxcl8, is a chemokine. Chemokines are chemoattractant that functions in directional recruitment of migrating immune cells. This includes lymphocytes, dendritic cells, neutrophils, NK cells etc. which moves through chemotaxis (movement induced by chemical stimuli) (69). IL-8 is proven to be important for neutrophil recruitment and guiding in zebrafish as well as the speed of migration, indicating a crucial role in early innate immune response (70).

Interleukin 12

IL-12 are pro-inflammatory cytokines important for type I helper T cells (Th1) (71). In humans, IL-12 are secreted by APCs and are an important bridge between the innate and adaptive immune system, as they initiate the differentiation of naïve CD4+ T cells into memory T-cells and cytokine-producing Th cells (72). IL-12 is a heterodimeric (contains two protein monomers) cytokine. In humans, IL-12 is comprised of subunits p35 (α-chain) and p40 (β-chain) (73). Characterization of the function of teleost IL-12 is still poorly understood. However, several p35 and p40 subunits are identified, and are most likely present due to the whole genome duplication seen in fish, and might therefore be able to create several IL-12 isoforms with different functions (74). Though the function of IL-12 is not fully understood in teleost, research indicates that IL-12 subunits possibly possess the ability to function as both homo- and heterodimers. IL-12 is believed to be related to Th cell response and is most likely in fish part of a greater internal regulatory network, as the different IL-12 combinations available might create additional competition in receptor binding when several IL-12 family cytokines are present (75).

Interleukin 10

IL-10 is an immune suppressive cytokine with anti-inflammatory properties. This cytokine is known in humans to inhibit synthesis of several cytokines as IL-1β, IL-8 and IL-12 from monocytes and macrophages. In general, it suppresses multiple immune responses through interacting with other immune cells (76). IL-10 homologs have been found in several fish species and structural features indicates close functional similarities to the human version (77). A study investigating the effect of IL-10 on lymphocytes and phagocytes in carp showed typical inhibitory activities. This was evident by the deactivation of neutrophils and macrophages and reduction in expression of pro-inflammatory genes as well as *mhc* genes involved in antigen presentation. IL-10 also showed to have regulatory effects on T cells as well as effects on

stimulation, proliferation and antibody secretion in B cells (78). However, studies of IL-10 expression in rainbow trout and carp has suggested that this interleukin acts as an inflammatory cytokine in fish, rather than an immune suppressive (77, 79). This was based on how quickly the expression levels of IL-10 was seen after lipopolysaccharide (LPS) stimulation, a trait common for inflammatory cytokines as IL-1β and IL-8. Due to the lack of substantial proof of this hypothesis, IL-10 is in this thesis considered to act as an anti-inflammatory cytokine.

1.4.2 The immune system of cod

Though the immune system of cod shares several similarities to other teleost, there are a few differences that are worth pointing out. MHC class II are proteins in the outer membrane of APC. These antigen presenting structures are important for triggering a wide adaptive immune response by stimulating CD4+ T-cells. Cod do not possess active genes for either MHC class II nor CD4. However, an increased amount of MHC class I genes and an extraordinary composition of TLRs have been described, which is proposed to compensate for this absence (80). MHC class I are proteins present on all nucleated cells and like the MHC class II, are antigen presenting complexes. MHC class I activates binds to receptors on cytotoxic T cells (TCR and CD8), and stimulates destruction of the presenting cell. TLRs are present in membranes of phagocytic cells and binds to PAMPs, which stimulates cytokine secretion from the cell. In addition, cod has shown to have a higher serum concentration of IgM compared to other teleost (81). IgM is an Ig that is linked to the primary immune response, and one of the first antibodies to appear in response to antigen exposure (62). Vaccine experiments on cod has shown that antibody response in general are weaker and that secondary immune responses does not evoke a greater antibody titer, as one would expect from ‘normal’ immunological memory. However, a protective effect is seen after vaccination, indicating that cod immunity may be different, but very much functional (82). Overall, this affects the strategy of vaccine development for species such as cod, since common vaccine technologies used, rely heavily on antibody response.

1.5 Piscirickettsiosis: *Piscirickettsia salmonis*

Piscirickettsiosis, also referred to as salmon rickettsial septicemia (SRS), is a life threatening condition caused by the Gram-negative bacteria *Piscirickettsia salmonis* (*P. salmonis*) in several salmonid species, among them Atlantic salmon (*Salmo salar*). *P. salmonis* are pleomorphic but most often seen as coccoid (size ranging from 0.5-1.5 μm in diameter), non-motile, non-encapsulated bacteria (38, 83-85). It was initially believed to be obligate intracellular but were later proven to be facultative intracellular due to it being able to be cultured *in vitro* on both agar plates and in liquid medium. In addition, the acquisition of the disease is mainly accomplished by horizontal transmission, unlike the *Rickettsia* (class α-Proteobacteria) which is mainly transmitted via vectors and are obligate intracellular (84, 86-88).

Even though *P. salmonis* is referred to as rickettsia-like, this is only due to morphological similarities between this bacteria and members within this family, as 16S rRNA gene sequencing puts the *P. salmonis* in the order Thiotrichales (class γ-Proteobacteria). This places *P. salmonis* phylogenetically close to the genera *Coxiella* and *Francisella* (both in class γ-Proteobacteria) and are expected to share more similar characteristics to strains within these genera than with the *Rickettsia* (class α-Proteobacteria) (13, 38, 89). In rainbow trout (*Oncorhynchus mykiss*), it was discovered by Smith et al. that *P. salmonis'* way of entry is most likely through skin and gills, as oral administration was proven less effective due to the acidic environment and digestive enzymes in the stomach of the fish (38, 90).

Even though the pathogen has been isolated from salmonids in the freshwater stage of its life cycle (91, 92), the severity of the disease, increased mortality and spread is linked to the transfer from freshwater habitat to seawater, with symptoms emerging 6-12 weeks after transfer (93). Even though little is known about the infectious route of *P. salmonis*, it has been proven that the bacterium replicates within membrane-bound cytoplasmic vacuoles in host cells, most commonly in macrophages (84, 94).

1.5.1 Symptoms and diagnosis

Piscirickettsiosis is diagnosed based upon external, internal, and behavioral signs characteristic for the disease. However, symptoms are heavily dependent on the degree of infection. While newly infected fish may show no external signs of the disease, moribund fish may have a darker appearance, be anorexic and lethargic, and swim near the surface and edges of its cage. The most consistent sign of this disease is the appearance of pale gills in relations to significant anemia, and abdominal swelling due to an enlarged spleen and swollen kidney. A range of skin lesions has also been observed, from raised scales to hemorrhagic skin ulcers. Even though a range of different symptoms are related to the onset of this disease, sudden death may occur during acute infections, without any gross signs of the disease.

The severity of the disease can also be determined looking at the tissue on a microscopic level. Lymphoid tissue in the spleen and hematopoietic tissue in the kidney are often replaced with diffuse chronic inflammatory tissue, and necrotic cells are seen scattered, but closely associated to vessels with degenerative changes. Necrosis of the hematopoietic cells are often followed by a granulomatous inflammation which in turn is responsible for the anemic condition of the fish. *P. salmonis* is commonly observed within macrophages because it commonly infects and replicates within these cellular compartments, which is why this pathogen is described as facultative intracellular. *P. salmonis* can be both observed and cultured from spleen, kidney, liver, gills and muscle of infected fish (13, 38, 87, 93, 95).

1.5.2 *Piscirickettsia salmonis* strain specificity*:* LF-89 vs. EM-90

The first strain of *P. salmonis* was characterized in 1989 by Bravo et. al as the etiological agent of the epizootic disease among coho salmon (*Oncorhynchus kisutch*), were designated strain LF-89 in 1992 by Fryer et. al (84). At the time of writing, there are several isolated strains of *P. salmonis*, but for the purpose of this thesis, only the strains LF-89 and EM-90 are discussed, as both were used in the vaccine trial analyzed (see section 2.1). Both strains were isolated from infected fish from Chile during outbreaks in coho salmon (1989) and Atlantic salmon (1990) (89). LF-89 and EM-90 differ by 22 nucleotides in the 16S gene, corresponding to a 98.5% similarity. Based on analysis of 16S rDNA, ITS and 23S rDNA, Mauel et. al showed in 1999 that between six isolates of *P. salmonis* from different geographical areas (Chile, Norway, Canada), EM-90 were the most genetically divergent compared to the other strains (Figure 1)

(89). This divergence is theorized to be due to the difference in presence of secretion systems and other virulence-associated genes in the two isolates.

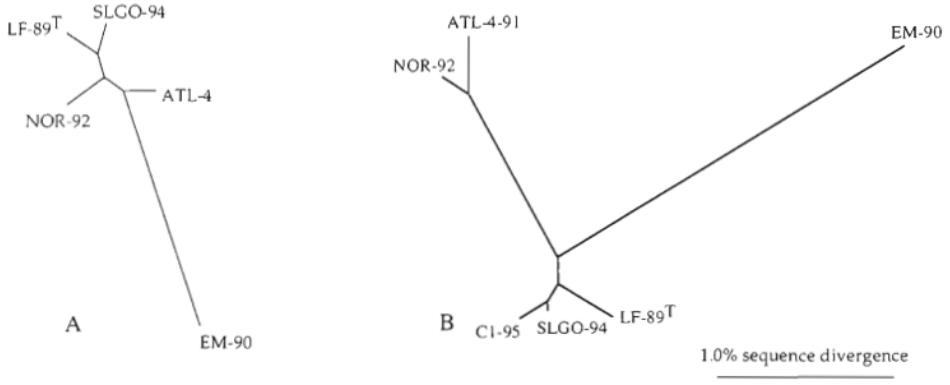


Figure 1 Phylogenetic relationships between six different strains of P. salmonis. A: 16S rDNA analysis. B: internal transcribed spacer (ITS). Both analysis shows that *P. salmonis strain EM-90 share less sequence similarities compared to the other strains (adapted from Mauel et al. (89)).*

Research by Gómez et. al characterized the presence of four functional type IV secretion systems (T4SS) in LF-89, and in addition confirmed their importance in virulence and pathogenesis in this strain (96). However, a comparative analysis performed on the EM-90 revealed only two T4SS, which was missing ~30 kb compared to the complete T4SS seen in LF-89 (97). In addition, an indel encoding *tra* genes was only detected in LF-89. The two strains also show differences in susceptibility to several antibiotics, where only EM-90 was susceptible to oxolinic acid and only LF-89 being susceptible to flumequine (98).

In a study performed by Rozas-Serri et al. in 2017 by cohabitant challenge in post-smolt Atlantic salmon, a difference in pathogenesis was seen between the two strains. The LF-89 strain showed lesions in liver and kidney, while the EM-90 strain showed a greater cumulative mortality with a more systemic and hemorrhagic disease, with lesions in brain, heart, kidney, liver, skeletal muscle and intestine (99). In addition to the higher cumulative mortality, the onset of death appeared earlier for the EM-90 cohabitant fish (36 vs. 40 days post infection), and the overall mortality was higher (94.4% vs. 81.7% in LF-89)(99). However, it is important to recognize the effect of serial *in vitro* passage and how this may have affected the virulence of LF-89 (strain retrieved from a reference laboratory in Chile) (97).

1.5.3 Current treatments and antibiotics

There are several vaccines against piscirickettsiosis on the market in Chile, though their efficacy has not led to any revolutionary disease preventions (100). According to Kevin Maisey et al., there were in 2016, according to the Servicio Agrícola y Ganadero, 33 available vaccines against piscirickettsiosis (38). Since very little is known about how *P. salmonis* infects and spreads once transmitted, the most common formulation used consists of bacterin (31 of 33 vaccines available) (101). One of the problems with the use of bacterin is the fact that it mainly provokes a humoral immune response, with the production of long-lasting plasma cells (101, 102). *P. salmonis* is mainly found intracellularly during an infection, unlike antibodies that circulates extracellularly. Even though an antibody response may elicit a positive effect during early infection where the bacteria may exist outside the cell before entry, very little is known about how *P. salmonis* continuously spreads throughout the fish. The efficiency of antibodies provoked from a vaccine based on bacterins would very much depend on this transfer from its primary multiplication site to the next. This would explain the low levels of consistent protection, if the pathogen were to be able to spread without entering the extracellular space (101). For this reason, the need for vaccines that specifically provokes the cellular immune response may be of importance, as well as characterizing the infectious route of *P. salmonis* in Atlantic salmon in detail. An example of an available vaccine against piscirickettsiosis is Birnagen Forte® 2, which was introduced to the marked in 2007. This vaccine consists of subunits Hsp60, Hsp70 and FlgG, which was proven to reduce the cumulative mortality in Atlantic salmon infected with *P. salmonis* strain LF-89. However, efficacy duration is estimated to only be 18 months (38, 103).

Due to the lack of long-term effective vaccines, antibiotics is still a common part of preventative and curative regimes used against piscirickettsiosis in Chile and was in 2014 reported to account for 90% of the total amount of antimicrobials used in Chilean aquaculture (13, 19, 38, 104). Even though *P. salmonis* has shown to be susceptible to several antibiotics *in vitro* (Table 1), treatments remains mainly ineffective due to the intracellular lifestyle of the pathogen, suboptimal concentrations of the antibiotic with oral administration, and possible inhibition of the antibiotics due to cations in seawater (105, 106).

Table 1 Shows In vitro minimum inhibitory concentration (MIC) for select antibiotics values against P. salmonis strain LF-89

Antibiotic	Minimum inhibitory concentration (MIC)	Susceptible/ Inhibition	Year tested	Reference
Streptomycin	100 µg/mL	Yes	1990	(83)
Gentamicin	50 µg/mL	Yes	1990	(83)
Tetracyclin	15 µg/mL	Yes	1990	(83)
Penicillin	100 IU/mL	No	1990	(83)
Oxilinic acid	2.5 µg/mL	No	1996	(98)
Flumequine	0.1 µg/mL	Yes	1996	(98)
Oxytetracycline	0.06 µg/mL	Yes	2018	(107)
Florfenicol	0.25 µg/mL	Yes	2018	(107)
Chloramphenicol	0.5 µg/mL	Yes	2018	(98, 107)
Streptomycin	8.0 µg/mL	Yes	2018	(107)
Sulfamethoxazole/ trimethoprim	512 µg/mL	No	2018	(107)

The main antibacterial agents used to combat piscirickettsiosis in Chile are flumequine, oxolinic acid, oxytetracycline and florfenicol (100). The quinolones flumequine and oxolinic acid used to be the initial choice of treatment, but was in 2007 determined as a "critical important antimicrobial for human medicine" by the World Health Organization (WHO), and has since decreased to less than 1% of total use (108, 109). FAO suggested in 2012 that *P. salmonis* should be treated as clinically resistant due to the relative effectiveness of antimicrobial agents, which increases the need for better vaccines with long-term effects (100).

1.5.4 Membrane vesicles

A common trait among bacteria is the ability to produce membrane vesicles (MVs). MVs are spherical structures that originates from membrane-enclosed areas. The content of MVs are highly variable and may contain signal molecules, lipopolysaccharides (LPS), metabolites, etc. MVs are involved in several functions such as pathogenesis, communication, biofilm formation, and DNA transfer (110). The lipid and protein content of MVs can provide information about its origin as the composition will resemble the membrane from which it originated (111). The Gram-negative cell wall consist of an outer membrane, a network of peptidoglycan and an inner membrane. The compartment between the inner and outer membrane is called periplasm (12).

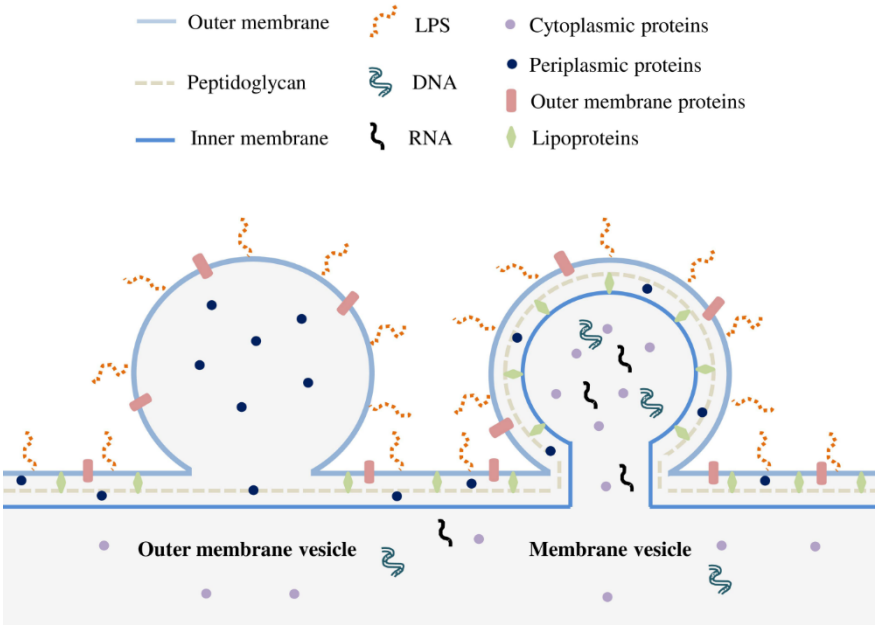


Figure 2 Outer membrane vesicles vs. outer-inner membrane vesicles (denoted membrane vesicle). Illustration by Tandberg J. (113).

Due to the presence of two separate membranes, Gram-negative bacteria are able to create two distinct MVs. Membrane vesicles described from Gram-negatives are mainly referred to as outer membrane vesicles (OMVs). As its name indicates, these vesicles only contains one lipid bilayer, which originates from the bacterial outer membrane. However, studies examining membrane vesicles retrieved from Gram-negatives in a transmission electron microscope (TEM) have revealed a second type of OMVs containing a double-bilayer. These MVs consist of both the inner and outer membrane and are referred to as outer-inner membrane vesicles (O-IMVs). The content of these MVs are highly variable, as OMVs contains periplasmic content and O-IMVs contains cytoplasmic content, such as DNA (Figure 2) (110, 112, 113).

Like several Gram-negative bacteria, *P. salmonis* has also been shown to produce membrane vesicles (114). A protein commonly found within MVs of *P. salmonis* is the chaperonin GroEL, which assist in folding of denatured proteins (115, 116). This protein, which is also found within MVs of *Francisella noatunensis* (117) is a well-known bacterial antigen, which has shown to induce both adaptive and innate immune responses (115). A study by Tandberg et. al showed that difference in strain virulence of *P. salmonis* could possibly be attributed to differences found between the MV contents. When comparing two less virulent strains from Norway and Canada (NVI 5692 and NVI 5892, respectively) and strain LF-89, a difference in protein content and volume were found, including GroEL being more abundant in strain LF-89 (116).

Due to the fact that little is known about the virulence mechanism of *P. salmonis*, both secretion systems and MVs has been suggested as mechanisms to deliver virulence factors. Oliver et. al showed in 2016, using *P. salmonis* strain LF-89, that it not only produced MVs, but also induced a cytopathic effect when adding purified MVs from *P. salmonis* to CHSE-214 cell line from Chinook salmon (*Oncorhynchus tshawytscha*). These finding suggest that *P. salmonis* may utilizes MVs as vehicles for virulence delivery and the fact that the MVs themselves are biologically active and partaking in *P. salmonis* pathogenicity (114). In addition to GroEL, the presence of several other virulence factors related to pathogen survival, antibiotic resistance, host invasion and intracellular trafficking in *P. salmonis* MVs, including hemolysin and outer membrane porin F. The presence of these pathogen associated molecular patterns (PAMPs) highlights its potential as a vaccine candidate against piscirickettsiosis, as PAMPs are effective in eliciting an immune response (118). Though the name indicates that PAMPs are only present in pathogens, PAMPs are present on all microbes. These molecular motifs include LPS, flagella, CpG-rich DNA and other shared traits within a class of microbes. PAMPs are recognized by TLR on several phagocytic cells and is part of the early acting innate immune response (63).

The effectiveness of MV based vaccines has been shown for several other diseases, both in humans as well as in other fish species. An example is the vaccine against *Neisseria meningitides. N. meningitides* is the common cause of bacterial meningitis, a severe inflammation of the meninges covering the brain and spinal cord of humans. The current available vaccine in Norway, Bexsero®, is partly based on MVs harvested from *N. meningitides.* Another example is a study by Brudal et. al, who utilized MVs from *Francisella noatunensis*, which showed a protective effect in zebrafish when immunized with 40 µg MV, followed by a lethal injection of *F.n.n.* (117). In conclusion, both present available vaccines and previous

trials in zebrafish shows that MVs are interesting and potential vaccine candidates, that is worth further investigation.

The efficiency of MVs as a possible vaccine candidate has earlier been attempted and proven efficient in a zebrafish model against *P. salmonis* infections. MVs were harvested from *P. salmonis* strain LF-89 and administered by intraperitoneal injection (injection into body cavity). The MV immunized group showed a cumulative survival of 84.2% compared to only 21.4% in the control group after injection with a lethal dose of *P. salmonis.* In addition, several immune related genes were upregulated as well as a reduced load of bacteria were seen in tissue (119). In summary, the study showed that an MV based vaccine shows promising results.

1.6 Francisellosis: *Francisella noatunensis* subspecies *noatunensis*

Francisella noatunensis subsp. *noatunensis* (*F.n.n.*) are part of genus *Francisella*, a group of pathogenic Gram negatives, from where *F. tularensis* (causative agent of tularemia/rabbit fever in humans) is widely known. Genus *Francisella* has been divided into two main clades composed of both terrestrial and marine occupying species, but analysis suggests that all species originates from marine habitats (120). The current clades separates the *F. tularensis* and *F. noatunensis*, which is supported by the independent development of host adaption, as *F. tularensis* and *F.n.n.* mainly infects mammals and fish respectively. However, both *F.n.n.* and *F.t.t.* has shown to be highly clonal, unlike the significant recombinant frequencies seen in some *Francisella (120). F. noatunensis* is further divided into two groups *F.n.n.* and *Francisella noatunensis* subsp. *orientalis* (*F.n.o*). However, whole-genome analysis has proposed that although they share a common ancestor, these represent a paraphyletic group, though this still remains to be confirmed (120). Nevertheless, this hypothesis is further strengthened when considering host-specificity, as *F.n.n.* is generally isolated from cold water fish species like cod and salmon, while *F.n.o* is isolated from warm water fish species such as tilapia (120).

Francisella sp. is classified into the class γ-proteobacteria, order Thiotrichales, like *P. salmonis*, which is a distant relative species, though genetically related (120). The *F. noatunensis* are non-motile, aerobic, coccobacillus that like *P. salmonis*, are facultative intracellular. Another trait shared with *P. salmonis* is the capability of phagosomal escape, a crucial ability for cytosolic replication (121). As *F. tularensis* and *F.n.n.* are closely related species, it is not uncommon that they share similar traits for successful cytosolic replication and/or virulence factors.

1.6.1 Disease and transmission

Francisellosis is highly infectious and easily recognized and diagnosed by the presence of characteristic multi-organ granulomas (14). The disease has been observed in several fish species, as Atlantic salmon in Chile (122), Atlantic cod in Norway (123) as well as tilapia. Infected cod show behavioral signs such as reduced swimming performance and appetite, as well as the presence of white granulomas both externally and internally (123). Mortality varies among species and is highly affected by water temperatures and presence of coinfections (14, 124). In Norway, it is characterized as one of the most serious diseases among Atlantic cod and currently considered a "List 3" disease by the Norwegian food safety authority. This list consist of diseases that are to be reported immediately on suspicion or occurrence of an outbreak (125).

A study on the prevalence of *F.n.n.* among wild cod along the Norwegian coastline conducted by Ottem et. al, showed that the overall prevalence was 13%. Interestingly all cod that showed presence of *F.n.n.* were located south of 'Sogn og Fjordane', which is consistent with the fact that water temperature is an important environmental factor for disease severity and occurrence (126). In general, higher water temperatures are related to increased disease occurrence and mortality (14). Even though francisellosis is characterized among Atlantic salmon in Chile, it is still to be identified in Norway, which may indicate a difference in host affinity among the north-Atlantic strain of *F.n.n* (14).

Even though the exact route of transmission of *F.n.n.* is not known, there are several theories and possibly several ways the pathogen may spread. Fish used by brood fish companies are often caught in the wild, which may already be exposed to an infection (123). *F.n.n.* has also been identified in eggs from Atlantic cod, which suggest a possible vertical transmission (transfer from mother to offspring), though this is not confirmed (cited by Colquhoun and Duodu (14)). It is also proposed that horizontal transmission (transmission between individuals) may both be conducted by naturally occurring *F.n.n.* in aquatic environments, as well as from being transferred by vectors (123, 127). A fecal-oral route has also been proposed as a possible route of transmission, as *F.n.n.* could be cultured from 50% of cohabitant Atlantic cod gut (128). However, *F.n.n.* has shown to be non-cultivable when retrieved from seawater, and though they are still viable, they are no longer pathogenic to cod in this form (129).

1.6.2 Virulence factors and vaccine development

No effective vaccine against francisellosis is currently available (14). *Francisella* are highly infectious and are the cause of substantial economic loss in both cod and tilapia aquaculture. A crucial aspect of vaccine development is identification and characterization of factors important for invasion, growth, and virulence. This may not only contribute to vaccine development for each separate *Francisella* species, but also highlight structural similarities or dissimilarities within the genus. This can in turn improve the understanding of how the different host affinities have come to be and shed a light on a possible link between shared virulence traits and the highly infectious nature seen across all pathogenic *Francisella*.

It is already established that most *Francisella* share genetic similarities, as the *Francisella* pathogenicity island (FPI), an important cluster of 16-19 genes including *iglC* and *iglD*, two proteins related to intracellular growth and phagosomal escape (130-132). *iglC* together with *iglA* and *iglB* (also localized on the FPI) are some of the genes that has been proposed to be part of a type VI secretion system in *Francisella* (133). Even though the function of several FPI proteins still remains unknown, several studies has showed that mutation or deletion of several of the genes present in the FPI, has led to both reduced virulence and diminished intracellular growth (132, 134).

A type IV pili (Tfp) has been characterized in *Francisella tularensis* subsp. *tulatensis* (*F.t.t*). Tfp is a well-known structure among several Gram-negative bacteria (as well as Gram-positive) (135). Because *F.n.n.* and *F.t.t.* share other similar virulence factors and high infectivity, it has been proposed that a Tfp system may be present in *F.n.n.* as well. A study by Brudal et. al showed that several of the Tfp subunits found in *F.t.t.* (136) were present in *F.n.n.* However, several subunits were only present as pseudogenes (Figure 3) (137). Type IV pili are flexible, protein-based appendages that protrudes from the bacterial surface. These structures are linked to several different functions, as motility, adherence, immune escape, transformation, etc. (138). Interestingly, type IV pili show remarkable similarities to the type II secretion systems in Gram-negative, an apparatus known to secrete toxins (139).

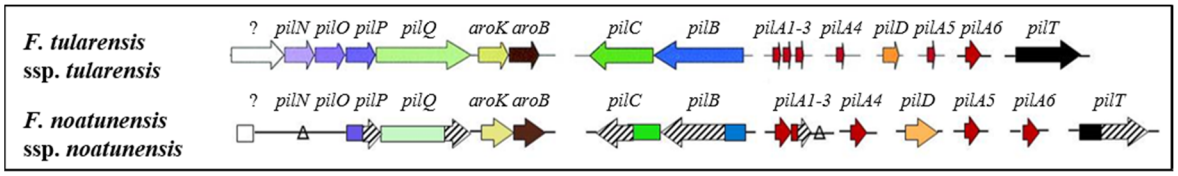


Figure 3 Genomic organization of Tfp system in Francisella strains (modified from Brudal et. al (137)). See Table 2 for Pseudomonas aeruginosa nomenclature. Hatched arrows mark pseudogenes. Open reading frame is indicated by arrow direction.

Table 2 Nomenclature and protein components of the type IV pilus. Table adapted from Melville et al. (135).

Protein category	Nomenclature*
Major pilins/pseudopilins	PilA
Minor pilins/pseudopilins	FimU, PilV, PilW, PilX, PilE
Assembly ATPase	PilB
Inner membrane core protein	PilC
Prepilin peptidase	PilD
Outer membrane secretin	PilQ
Retraction ATPase	PilT, PilU
Inner membrane accessory protein	PilM, PilN, PilO

*Nomenclature is from *Pseudomonas aeruginosa*

Type IV pili systems in Gram-negatives spans across both membranes and the periplasm (Figure 4). The main components consist of an assembly and retraction ATPase and prepilin peptidase as well as major and minor prepilins and membrane secretion/channel components (see Table 2 for nomenclature). The PilD protein is responsible for cleavage of the signal sequence on the prepilins and with help from the ATPase the pilins are assembled and protruded through the membrane-spanning secretion channel (135). Brudal et al. found that several genes encoding subunits of the type IV pili system were intact in *F.n.n.* (Figure 3). This included the PilD subunit and several prepilins (137).

An efficient way of deducing the role of a specific gene is to disrupt it by creating a mutant version. By removing the transcription possibility of a protein, it is possible to evaluate the overall response, by assessing the behavioral function and level of pathogenicity compared to the wild strain.

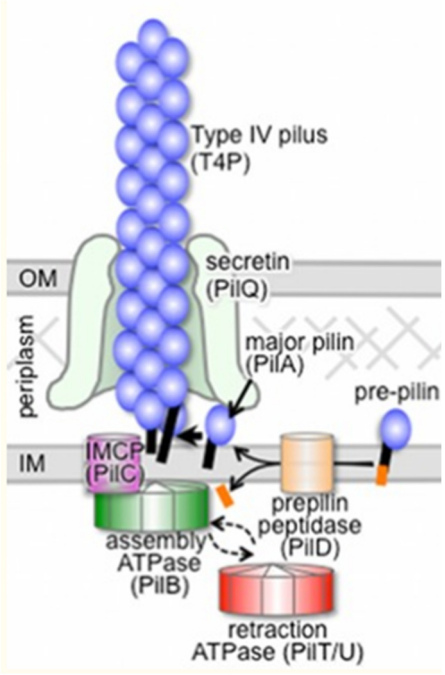


Figure 4 Gram-negative Type IV pili (modified from (135)). Nomenclature is from P. aeruginosa (see Table 2).

It has previously been proven in other Gram-negative with type IV pili, that mutation of apparatus subunits leads to reduced function or virulence (140-142). By mutating the subunits present in *F.n.n.*, it may be possible to determine why only some subunits of the pili apparatus are present, and their function. Since *F.n.n.* does not possess a functional genetic version of all the components needed for a complete type IV pili apparatus (137), as we know so far, it is interesting to both evaluate the presence of pili on the outer surface, and how, if present, these pili are affected by mutations in intact subunits. Pili are important virulence factors, and has been proven to induce a protective effect against both Gram-positive and –negative bacteria when given as a vaccine (143, 144).

A vaccine against tularemia, a severe human disease caused by *F.t.t.* is currently under development. Both inactivated whole-cell and live attenuated vaccines has been tested, with the live attenuated producing a greater protection compared to the inactivated vaccine (145). This live attenuated vaccine is derived from *Francisella tularensis holarctica* (*F.t.h.*), a less virulent strain of *F. tularensis*, also referred to as type B (146). The Tfp subunit PilA1 has been proven to be an important virulence factor in *F.t.h.* (147). The attenuation in the current live attenuated vaccine based on *F.t.h.* has been proposed to be linked to a deletion in a pili building block protein, PilA1 (148-150). In addition, deletion of both *pilF* and *pilT* were shown to affect pili production in the live attenuated vaccine strain. This strain has been shown to possess Tfp-like structures on its surface, in which *pilF* and *pilT* mutations resulted in loss of these surface appendages (142, 151). However, though there is only a few successful verifications of extracellular Tfp-like appendages on the surface of *Francisella*, secretion of pilin protein pilA has been shown to be transported to the surface of *F. tularensis* without the presence of definite pili (147). This increases the interest in characterizing the potential function of a Tfp system in *F.n.n,* as it may be useful in creating a "knock-out" version, and possibly give rise to an attenuated version of *F.n.n.,* as seen for *F.t.h.*

1.7 Aims

The aim of this thesis is to analyze the efficacy of a vaccine based on MVs from *Piscirickettsia salmonis* in Atlantic salmon, as well as evaluating the presence of a type IV pili system in *Francisella noatunensins* subsp. *noatunensis.*

A vaccine trial was conducted in Atlantic salmon using MVs harvested from *P. salmonis.* The goal of the vaccination was to immunize fish and prevent the onset of piscirickettsiosis. However, results showed no protection in terms of cumulative mortality. This thesis will assess the efficacy of the vaccine trial, by evaluating both the genetic expression of immunological genes as well as presence of disease markers in tissue. The goal of these experiments was to appraise any differences and possible protective effects seen at a microscopic level between the immunized groups. These results may help in improving future immunization trials utilizing MVs.

Previous studies have shown presence of several intact type IV pili system genes in *F.n.n.* As type IV pili systems are known virulence factors, in which mutation has led to attenuation of virulent strains, it is of interest to assess the presence of these components in *F.n.n.* Characterizing presence of functional pili may both aid in further understanding of the virulence mechanisms of *Francisella,* as well as contributing to further vaccine development.

The individual aims may be summarized as:

1. Evaluate the immune response of a selection of immune genes from an MV-based vaccine trial performed in Aet tlantic salmon against *P. salmonis* infection.
2. Assess the presence of disease markers in *P. salmonis* infected Atlantic salmon by histological examination after an MV-based vaccine trial.
3. Evaluate the presence of a type IV pili system in *F.n.n.* by examination in a transmission electron microscope and atomic force microscope.
4. Evaluate the presence of *F.n.n.* with positively labeled type IV pili system components by the use of a flow cytometry
5. Sort the positively labeled *F.n.n.* and appraise any fold increase/decrease in pili presence by the use of a flow cytometry.

2 Material and Methods

2.1 *Piscirickettsia salmonis*

A pilot laboratory efficacy trial was performed during the months of March to July 2016 (VESO vikan study number: V-3566) under the auspices of the University of Oslo. The purpose of the study was to test the efficacy of membrane vesicles harvested from *P. salmonis* (strain LF-89) as a vaccine in Atlantic salmon. Fish were vaccinated at time point 0. Fish were kept in salt water (12-35‰) before and during challenge. Light conditions before and during immunization were 12 hours of light and 12 hours of darkness. During and after smoltification, fish were kept in light at all times. Seven weeks after vaccination, fish were infected by cohabitation challenge with non-vaccinated fish injected with a 0.1 mL dose of *P. salmonis* EM-90 with an approximate concertation of 3.0×10^5 CFU / mL, to mimic a natural spread of infection (termed shedders). Spleen, liver, and kidney tissue from six fish in each trial group were harvested and stored in RNAlater for use in qPCR analysis at both 0 weeks post challenge (WPC) and 4 WPC and stored at -20°C. As high mortalities were observed in all groups (Figure 5), the trial ended at 4 WPC. Spleen and kidney from six fish in each trial group were harvested and stored in buffered formalin at both 0 WPC and 4 WPC for use in histologic analysis and stored at room temperature. Gill, muscle, liver and heart were also harvested but not used for this thesis. Buffered formalin was exchanged with 70% ethanol for long-time storage. Tissues harvested at 0 WPC were from fish that had not been challenged. See Table 3 for vaccine groups.

Table 3 Vaccine groups from V-3566 vaccine trial.

Group	Vaccine	Dose (mL)	Total no. fish
1	40 µg MV + Adjuvant*	0.1	65
2	Adjuvant* only	0.1	65
3	Control (0.9% NaCl).	0.1	65
4	Untreated	0.1	65
5	100 µg MV	0.1	65
6	Shedders	0.1	65

*The adjuvant used was Montanide ISA 780 VG.

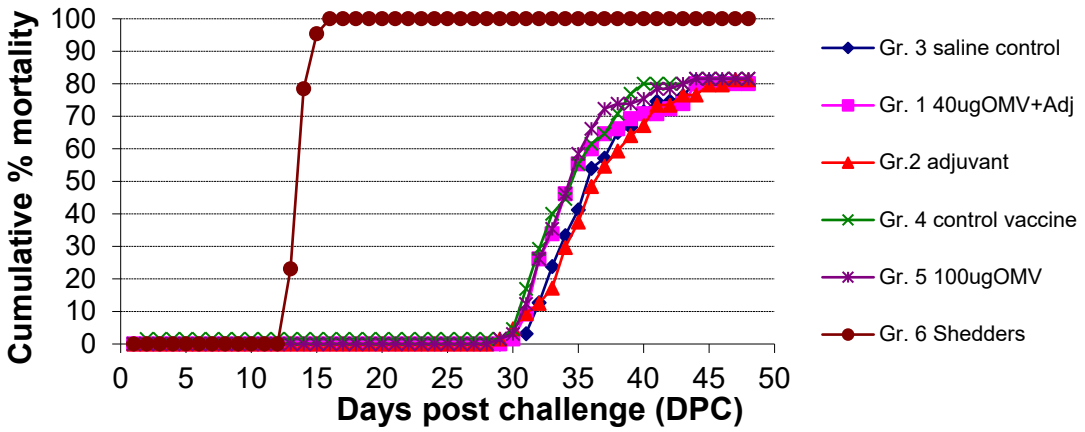


Figure 5 Cumulative mortality during vaccine trial of Atlantic salmon (2016) within the given groups. Efficacy evaluation performed by Dr. Alexander Kashulin and represented in a Kaplan-Meier representation. As seen, the onset of death within the shedder group appeared at day 13 of trial and 100% mortality were seen at day 16. Little difference is seen within the other groups. One death was registered at day 2 within the untreated group, but proper onset of morality was not seen until day 30. Onset of death is also seen at day 30 for group 1, 2, 3 and 5. An approximate 25% mortality is seen at day 32 for group 1,3 and 5, and at day 34 for group 2 and 3.

The following protocols were used to extract the necessary information from the respective tissues, in order to make an analytic evaluation of the vaccines effect at an immunological and histological level.

2.1.1 RNA extraction from Atlantic salmon tissue

RNA extraction is a method for extracting the genetic content of interest by utilizing a series of specific steps to process a sample of animalic tissue and only retrieving the RNA. The method does not exclude between the different types of RNA and is therefore a total RNA extraction method. RNA extraction was done by using the RNeasy mini kit from QIAGEN. Protocol for RNeasey mini kit (QIAGEN) extraction is found in Appendix 1.

2.1.2 Reverse transcription of RNA to cDNA

Reverse transcription was performed by using the High-Capacity RNA-to-cDNA™ (Thermo Fisher). 300 ng of extracted RNA for each reaction. Positive reverse transcription reaction (contains enzyme for reverse transcription) were made by mixing 10 µL of 2X RT Buffer mix with volume of RNA equal to 300 ng, 1 µL 20X RT Enzyme mix and Nuclease-free water up to a total volume of 20 µL in a 1.5 mL Eppendorf tube. A negative reverse transcription reaction (does not contain enzyme for reverse transcription) was set up as a control in parallel for all samples. Nuclease-free water replaces the 20X RT Enzyme mix in these reactions. Samples were incubated at 37°C for 1 hour in a digital dry bath (Labnet), and reaction were stopped by increasing the temperature to 95ºC and incubated 5-10 minutes. All positive reverse transcription stocks were diluted 1:10 with nuclease-free water for use in qPCR.

2.1.3 Primers

Primers used in this study were chosen based upon previous studies and relevant articles. A list of primers used may be found in the following table below.

Table 4 Primers used for qPCR analysis of Atlantic salmon tissues.

Gene	Abbreviation	Forward 5'→3'	Reverse 5'→3'	Accession number	Ref.
Salmo Salar 18S rRNA	18S	TGTGCCGCTAGAGGTGAAATT	GCAAATGCTTTCGCTTTCG	AJ427629	(152)
Elongation factor 1 alpha mRNA	Efα1	CACCACCGGCCATCTGATCTACAA	TCAGCAGCCTCCTTCTCGAACTTC	AF321836	(152)
Interleukin-1 beta mRNA	IL1β	GGAGAGGTTAAAGGGTGGCG	TCCTTGAACTCGGTTCCCAT	AY617117	(152)
MHC-Sasa class II B	MHCIIB	ATGGTGGAGCACATCAGCC	CTCAGCCTCAGGCAGGGAC	X70166.1	(153)
Macrophage expressed 1	MPEG	GGCAACATCACCTACTCCATAA	AGGTTGTTCTTGGTGCTCTC	XM_014172502	(154)
Suppressor of cytokine signaling 3-like	SOCS	GGGAAGGCAGCAACATGAGT	TTTTGGTTGGCAGCCTGTTG	XM_014202622.1	(152)
Interleukin 8	IL-8	GGCCCTCCTGACCATTACT	ATGAGTCTACCAATTCGTCTGC	NM_001140710	(155)
Interleukin 10	IL-10	CGCTATGGACAGCATCCT	AAGTGGTTGTTCTGCGTT	EF165029	(155)
Interleukin-12	IL-12	CTGAATGAGGTGGACTGGTATG	ATCGTCCTGTTCCTCCG	BT049114	(155)

2.1.4 Primer efficiency

Primer efficiency evaluation is a method for assessing the efficiency of each primer set upon amplification in a quantitative-PCR instrument. This evaluation is based upon the sequential amplification of the chosen sequences, and if 100% efficient, the amplification volume should double with each repeated cycle (see section for thorough explanation 2.1.5). An efficiency test will give an indication of how good the initial primer design was or if any changes has to be made in the concentration of reagents or the reaction conditions. Common problems include the presence of primer dimers (primer sets that upon mixing bind to each other instead of the sequence of interest, because of sequence complementarity) or non-specific primer sets that will amplify sequences other than those of interest.

The commonly accepted range of efficiency of the primers evaluated is desired to be between 90-110% (156). The reason primer efficiency is able to exceed 100% is mainly due to polymerase inhibition. This is more prone to happen in more concentrated or contaminated samples. The cycle threshold (Ct) values of these samples will therefore be higher compared to the more diluted samples that may not be inhibited. Samples diluted in a 10-fold to each other should give a ΔCt value around 3.3 when there is a 100% amplification efficiency. The less diluted samples may therefore be closer in Ct value to the more diluted samples and give a difference in ΔCt values < 3.3. As inhibition may decline as samples are more diluted, the ΔCt value may again increase to around 3.3. This however flattens the curve and gives the impression that the efficiency is >100%, as the slope gives an impression that a ΔCt value of less than 3.3 is needed to double the template amount, and efficiency exceeds 100%.

Primer efficiency were calculated by running a standard qPCR protocol (see section 2.1.5) by using a serial dilution of the primers (example: 1:10, 1:100, 1:1000 and 1:10.000) in 2-3 technical replicates with the same cDNA for all reactions. The average of the collected Ct values for each dilution were plotted on a logarithmic scale using Excel and a linear regression curve generated through the data points were obtained. The gradient was used for calculating the efficiency by utilizing Thermo Fishers qPCR efficiency calculator (157). See Appendix 4 for individual efficiencies. Efficiencies that is not in the desired range from this experiment is most likely due to pipetting error, as primers have successfully been used in earlier articles. Further efficiency testing was performed and improved for some of the primers, but the remaining unsatisfactory results were not repeated as the qPCR machine broke down between the experiments. Changing to a new instrument would entail repeating all the qPCR analysis and it

was determined that the obtained results were sufficient enough for the purpose of this study, based on the reference articles. Primers that had an efficiency that were outside the desired range (90-110%), were omitted from this study (Appendix 4).

2.1.5 Quantitative-PCR

Quantitative polymerase chain reaction (qPCR), also referred to as real time PCR, is a method used to determine the quantity of a sequence of interest. Unlike standard PCR, qPCR is based on a sequential amplification were each cycle of amplification is monitored by measuring the fluorescence emitted by the fluorescent compound utilized (in this study SYBR® Green from Sigma Aldrich were used) which only interacts with double stranded DNA. The threshold is determined automatically and is 10-fold higher than the average baseline (background) measured. When emitted fluorescence in the independent wells reaches the threshold, a Ct value is given which indicates the amounts of repeated cycles needed to create a significant signal. A lower Ct value corresponds to a larger amount of sequence present, because a lower amount of cycle repeats is needed to reach the threshold. For this study's purpose, a relative qPCR analysis were performed, which in contrast to absolute quantification, is a method where the expression of each gene of interest is determined on the fold-difference compared to the chosen housekeeping/reference genes (156).

For each reaction, the reagents listed in Table 6 were added to each well. The qPCR analysis was performed in the Lightcycler®480 system (Roche Life Science), see Table 7 for qPCR cycler setup used. For primers see section 2.1.3. The negative control from all tissue samples were used to check for contamination for at least one gene (preferably housekeeping gene 18S). The melting curves obtained from each cycler run were checked for all samples and genes to determine the specificity of the amplification products and if any unspecific binding were present. Primers that did not yield a satisfactory melting curve were omitted from this study (Appendix 4).

Table 5 Primer mix. The following reagents were mixed to create the primer mix used in the qPCR reaction.

Reagent	Amount (µL)
Fw Primer (100 µM)	5
Rv Primer (100 µM)	5
dH_2O	90
Total	100

Table 6 Reagent and amount used for each qPCR reaction

Reagent	Amount (µL)
SYBR® Green (Sigma Aldrich)	5
Primer Mix	1
dH_2o	2
cDNA	2 (diluted 1:10, 1.5 ng/µL)
Total	10

Table 7 qPCR cycler setup.

Step	Step	C°	Time	Cycles
Pre incubation	1	95	5 minutes	1
Denaturation	1	95	10 seconds	45
Annealing	2	60	10 seconds	
Extension	3	72	10 seconds	
Melting curve analysis **Denaturation**	1	95	5 seconds	1
Cooling	2	65	1 minute	
Denaturation	3	97	Continuous	
Cooling	1	40	30 seconds	1

Program in qPCR was setup for a 20 µL reaction volume for all reactions. However, only a 10 µL reaction volume were used for all samples.

The average Ct value between the technical replicates for each biological replicate within a vaccine group were calculated (see formula 1). The normalization factor was determined by the reference genes delta-delta Ct values (see formula 2). The reference genes utilized were 18S and elongation factor 1α (ef1α). Reference genes are commonly used in qPCR as a normalization strategy and are genes that varies minimally between cells and under different conditions. As their expression remains mainly unchanged, the Ct values obtained can be used to account for the variations that occurs due to experimental imperfections (158).

Formula 1- Shows the delta-delta Ct method.

$$Relative\ fold\ gene\ expression = 2^{Ct\ of\ lowest\ value - Ct\ of\ another\ sample}$$

Formula 2- Shows how the normalization factor were determined using the two housekeeping genes.

$$Normalization\ factor = (Relative\ expression\ 18S * Relative\ expression\ EF1\alpha)^{(1/2)}$$

The delta-delta Ct values were divided by the normalization factor to create the normalized relative expression of each gene between the control and vaccine group. The normalized expression value from the control group were averaged. All normalized expression values from control and vaccine group were divided by this average, creating the normalized expression value relative to the control group for the vaccine group, and a value of 1 for the control group.

One-way ANOVA was used to evaluate statistically significant changes in relative gene expression across each group, followed by Dunnett's multiple comparisons test, with a pre-determined $\alpha = 0.05$. Adjusted p-values lower than α were considered statistically significant. GraphPad Prism 8 was used for graphing and statistical evaluation.

2.1.6 Histology

Histological analysis were performed to evaluate the difference between tissue samples from the vaccinated and unvaccinated fish both before and after challenge. The purpose of this experiment was to determine the effect of the vaccine given by comparing tissues from infected and non-infected fish, as the formation of granuloma is linked to progressed piscirickettsiosis. By comparing the tissue harvested at 0 WPC and 4 WPC we can determine if the vaccinated fish show the same or a different level of disease markers, as granulomas.

Spleen and kidney from the two MV vaccine groups (40 µg MV + adjuvant and 100 µg MV groups) and the control groups (saline) from both 0 WPC and 4 WPC were used. Three biological replicates were used for each group. Tissues from the same group and biological replicate were placed in a glass container and approximately 5-6 mL of 80% ethanol added, before incubating at a rolling shaker for 30-60 minutes. After incubation, ethanol was removed and replaced with 5-6 mL of 90% ethanol and incubated at a slow-shaker for 30-60 minutes. This was repeated with 96% and three times 100% ethanol, creating an increasing alcohol series, leaving the tissue dehydrated. After the last incubation of 100% ethanol, the ethanol was removed and replaced with 5 mL of Technovit® 7100 (Kulzer) solution mixed with Hardener I according to manufacturer's protocol. Incubation were performed overnight on a rolling shaker. Hardener II were mixed with Technovit® and Hardener I solution in a volume of 50 µL pr. 1 mL respectively, and 500 µL added to a cold histoform before adding the sample tissue. Another 500 µL of mixture were added on top of sample before leaving to harden at room temperature for 1 hour. After 1 hour, histoforms were placed in a 37°C incubator over night to further harden. A plastic histoblock were placed on top of each sample. Technovit® Universal Liquid were mixed with Techonvit® 3040 according to manufactures protocol, and mixture poured inside the histoblocks, attaching the histoblock to the resin block. Resin is an embedding material used to stabilize the dehydrated tissue by infiltrating the sample, which then hardens and makes sectioning in a microtome easy. After 15 minutes incubation, resin blocks could be removed by pliers.

Resin blocks were sectioned using a microtome. Sections were picked up by tweezers and put in a beaker of milli-Q water to fully unfold before being picked up using a glass slide. Glass slides were left to dry on a 37°C hot slide drying bench. Each section was 3 µm thick and separated by 12-50 µm to avoid overlap. Both toluidine blue and periodic acid Schiff (PAS) staining were attempted.

PAS staining

Sections were rehydrated in tap-water for 1 minute and then incubated in 1% periodic acid for 15 minutes at room temperature. Sections were then washed in milli-Q water for 1 minute 2-3 times. Incubation in Schiff reagent was then carried out at room temperature for 60 minutes in a covered container, due to light sensitivity. Sections were then washed in tap water for 10 minutes. Staining were performed by incubating sections in hematoxylin for 10 minutes. Sections were then washed in tap water for 15 minutes and in milli-Q water for 1 minute. Slides were then left to dry on a 37°C hot slide drying bench. When dry, glass slides were mounted by placing droplets of pertex close to each section and adding a sufficient amount of xylene before covering with a glass cover slide. Slides were left to dry in fume hood overnight.

Toluidine blue staining

Sections were rehydrated in tap-water for 1 minute. Slides were then incubated in toluidine blue solution for 1 minute. Sections were then washed in tap-water for 5 minutes. Another wash step was then performed in milli-Q water for 1 minute. Slides were then left to dry on a 37°C hot slide drying bench. When dry, glass slides were mounted by placing droplets of pertex close to each section and adding a sufficient amount of xylene before covering with a glass cover slide. Slides were left to dry in fume hood overnight.

2.2 *Francisella noatunensis* subsp. *noatunensis*

F. noatunensis subsp. *noatunensis* (*F.n.n.*) 5330 mutants from a former study by Espen Brudal were utilized to evaluate the presence of a type IV pili system in this organism. Brudal concluded his Ph.D. at the Norwegian university of life science in the year 2014. His research encompassed work on *Francisella noatunensis* disease models and prevention as well as virulence factors. This thesis continues on the work on virulence factors by Brudal. Mutants utilized for further investigation of the type IV pili system in *F.n.n.* are retrieved from former research on *F.n.n.* at the Department of Pharmacy, University of Oslo. Mutants utilized and their origin can be found in Table 8.

Table 8 Francisella noatunensis subsp. noatunensis (F.n.n.) mutants used in thesis.

***Francisella noatunensis* subsp. *noatunensis* mutants**	**Abbreviation**	**Plasmid content**	**Reference**
F.n.n 5330 pKK289km*: pilA1-C6His*	*F.n.n. pilA1:his*	Kanamycin resistance His-tagged prepilin gene *pilA1*	(137)
F.n.n 5330Δ*pilD* pKK214: *pilA1-C6His*	*F.n.n. ΔpilD pilA1:his*	Kanamycin and tetracycline resistance His-tagged prepilin gene *pilA1* Disrupted Tfp system component PilD	(137)
F.n.n pKK289: *gfp*	*F.n.n. gfp*	Kanamycin resistance Plasmid containtin gene encoding for green fluorescent protein GFP	(159)
F.n.n pKK289: *clpB*	*F.n.n. clpB*	Kanamycin resistance Plasmid containing the *clpB* gene	(160)
F.n.n pKK*pilA6-His*	*F.n.n. pilA6:his*	Kanamycin resistance His-tagged prepilin gene *pilE6*	(137)
F.n.n pKK286*pilA2-His*	*F.n.n. pilA2:his*	Kanamycin resistance His-tagged prepilin gene *pilE2*	(137)
F.n.n pKK286*pilA4-His*	*F.n.n. pilA4:his*	Kanamycin resistance His-tagged prepilin gene *pilE4*	(137)
F.n.n pKK286*pilA5-His*	*F.n.n. pilA5:his*	Kanamycin resistance His-tagged prepilin gene *pilE5*	(137)
F.n.n Δ*pilD* pKK214*pilA5-His*	*F.n.n. ΔpilD pilA5:his*	Kanamycin and tetracycline resistance His-tagged prepilin gene *pilE5* Disrupted Tfp system component PilD	(137)
F.n.n Δ*pilD* pKK214*pilA6-His*	*F.n.n. ΔpilD pilA6:his*	Kanamycin and tetracycline resistance His-tagged prepilin gene *pilE6* Disrupted Tfp system component PilD	(137)

2.2.1 Eugon Chocolate Agar (ECA) plates

ECA plates were utilized for growth of *F.n.n.* (as previously published by Espen Brudal (161)). 30,4 g/L Bacto™ Eugon Broth powder was transferred to a 1 L flask. 800 mL of dH_2O were added to the flask and heated until boiling on a hotplate under constant stirring with a magnet. Mixture was boiled for 1 minute. 200 mL of distilled water were added after boiling to a total volume of 1 L. 15 g/L of Agar bacteriological (AGAR NO.1, Thermo Fisher) was added to the mixture and autoclaved. Bovine blood was added aseptically to a total of 5% (20 mL to 1 L) directly after autoclaving. The flask was placed in a 56℃ water bath, shaking at a rate of 70 rpm for 30 minutes. The flask was removed from the water bath and 1 mL of 2 M $FeCl_3{}^x6H_2O$ was added, under a sterile hood, before placing back in water bath to keep media dissolved. Transfer of media too plates were performed under a sterile hood and plates kept at room temperature for 1-2 hours to cool down. To ensure conservation of the pKK plasmid and selective bacterial growth of *F.n.n.* strains, antibiotics were added (Table 9). When using kanamycin, the antibiotic was added directly to the media after removal from the water bath, and manually dispersed before transferring to plates. When using tetracycline, the antibiotic was not added to the mixture because of its heat intolerance. Tetracycline was added as described in 2.2.2.

Table 9 Concentration of antibiotics utilized for selective bacterial growth on plates.

Antibiotic	Stock concentration	Amount added	Concentration on plates
Kanamycin	50 mg/mL	500 µL in 500 mL ECA solution	0.05 mg/mL
Tetracycline	12 mg/mL	7.5 µL (added on top of dry ECA plate)	12 mg/mL

2.2.2 Plating of bacteria

All bacterial mutants used were stored at -80℃ in glycerol or skimmed milk stocks. Plating was performed by slightly thawing the bacteria and transferring with a sterile loop to an ECA plate. The plates were sealed with parafilm to avoid drying of the bacteria and kept at 20℃ for 4-7 days. When colonies were present on the ECA plates, they were transferred to new ECA plates containing antibiotic, for selective growth. The bacteria were in an earlier study transformed with the plasmid pKK to contain a gene of resistance to the respective antibiotics as listed in Table 9. If bacteria were to only be plated on kanamycin, the colonies were transferred to the

ECA plates containing kanamycin with a sterile loop, sealed with parafilm, and stored at 20℃. If bacteria were to be selectively grown on both kanamycin and tetracycline, 7,5 µL of tetracycline were added to an ECA plate containing kanamycin and spread with a sterile spreader. When dry, colonies were transferred to the tetracycline-applied area, sealed with parafilm, and covered with aluminum foil to avoid light due to tetracycline being sensitive to light, and stored at 20℃ for 4-7 days.

2.2.3 Liquid bacterial culture

Liquid cultures of bacteria were made to make the transfer of bacteria to formvar-coated copper grids during immunogold labeling and for use in flow cytometry (see section 2.2.7 and 2.2.10). Colonies of bacteria were transferred with a sterile loop to a 15 mL sterile plastic tube containing 5 mL Eugon broth supplemented with 5 µL of 2 M $FeCl_3$ (161). The antibiotics were added to ensure selective growth (Table 10). Cultures containing tetracycline were covered with aluminum foil to avoid inactivation of the antibiotic due to its light sensitivity. Cultures were incubated at 20℃, shaking, for 4-7 days.

Table 10 Concentration of antibiotics utilized for selective bacterial growth in liquid culture.

Antibiotic	Stock concentration	Amount added	Concentration in liquid media
Kanamycin	50 mg/mL	7.5 µL	0.08 mg/mL
Tetracycline	12 mg/mL	7.5 µL	0.02 mg/mL

2.2.4 Freezing of bacteria (-80 °C)

Bacteria from plate were scooped up using a sterile loop and dispersed in 500 µL of EB in a cryotube. 500 µL of 50% glycerol were then added to this solution and then left at -80 °C, for long time storage.

2.2.5 Formvar coated copper grids

Copper grids with 150 or 200 mesh, were placed in beaker with 100% acetone and put in sonicator under fume hood for 5 minutes. Girds were then dried by placing on a filter paper. Formvar solution were made by measuring the acquired amount of formvar to a 1-2% concentration and adding 50 mL of chloroform. Solution were covered and left overnight to dissolve while sonicated. Clean glass slides were added to a coating apparatus and incubated in the formvar solution for 2 minutes. Pre-adjustments were made to ensure the flowrate across the glass slide to be approximately 11 seconds, when opening the flow tap on apparatus. Glass slides were then dried at room temperature. Glass container were filled with milli-Q water. A single edge razor was used to cut along the edges on each side of the glass slide to ensure proper release of film. Breathing on both sides of the glass slide before vertically slowly dipping the slide into water left the film floating on top of the water. Grids were placed onto the floating film. Grids were picked up by "rolling" a piece of parafilm on top of film with grids and left in a petridish to dry.

2.2.6 Preparation of negative stain for transmission electron microscopy

Bacterial culture was picked from ECA plate using a sterile loop and transferred to Eppendorf tube with 20-80 µL 60 mM HEPES. Droplet of bacterial culture were placed on a parafilm and a formvar coated grid placed film-side down on droplet and incubated for 5-10 minutes. Washing were performed twice by moving grid to droplet with distilled water and left for 1 minute. Grid were moved to droplet of 1% uranyl acetate and left for 1 minute. Washing step were repeated twice. Grid were then dried and left in grid-storage box or coated by placing grid film-side down on droplet of 2% methyl cellulose and dried.

2.2.7 Immunogold

Immunogold labeling is a combined laboratory technique that utilizes the efficiency of an antibodies ability to directly bind to its target of interest while at the same time being bound to a gold particle. The gold particle can easily be detected due to its spherical shape and high electron density, using a transmission electron microscope. The method was in this study used for detection of a suggested extracellular type IV pili system structure of *Fransicella noatunensis* subsp. *noatunensis*. The bacteria have earlier been transformed to contain the mutations of interest, see Table 8. By utilizing the THE™ His-tag mouse monoclonal antibody (Genscript) and a bridging antibody Goat-anti-mouse IgG (H+L) (Invitrogen), the potential selectively binding of the antibody to the his-tagged prepilin structure of the *F.n.n* was determined. This was visualized by adding a protein A-gold (PAG) complex to the sample, which binds to the secondary antibody. PAG binds less effectively to antibodies from animals such as mouse and sheep, and is the reason for why a secondary antibody were utilized, as PAG binds more sufficient to goat antibodies.

All steps of immunolabeling were performed at room temperature. *F.n.n*. mutants were first revived from frozen storage condition and grown on ECA-plates without antibiotics. Colonies were transferred to ECA-plates containing antibiotic (kanamycin) to select for the *F.n.n* strains containing the pKK plasmids. At the day of immunolabeling, bacteria were transferred to Eppendorf tubes containing 0.5 mL EB or 60 mM HEPES buffer (HEPES powder dissolved in water, 0.1M). All steps were performed by placing the following reagent onto a sheet of parafilm and placing the formvar coated grid with formvar film side down onto droplet. Several protocols were attempted to optimize the specificity of the antibodies and the addition of positive and negative controls.

Transfer of bacteria to the grids were performed by either utilizing liquid bacterial culture, dispersing solid colonies of bacteria into Eugon broth/HEPES or directly placing the grids onto a solid colony. Grids were kept on bacterial solution for 5 minutes and then washed twice with 120 mM HEPES for 1 minute. Fixation were performed by placing grids on droplet of paraform aldehyde in HEPES (4% and 120 mM respectively) for 5 minutes. The grids were washed three times with 120 mM HEPES for 1 minute before transferring to droplet of 1% Fish-skin gelatin (FSG) to block non-specific binding sites, for 15 minutes. The grids were placed on droplet of mouse monoclonal antibody diluted 1:200 in 1% FSG for 30-45 minutes. The grids were washed by briefly passing a droplet of 120 mM HEPES and then five additional washing steps

with HEPES, each for 1 minute. The grids were then placed on droplet with secondary antibody, goat anti-mouse IgG diluted 1:200 in 1% FSG and incubated for 30-45 minutes. The grids were then washed by briefly passing a droplet of 120 mM HEPES and then five additional washing steps with HEPES, each for 1 minute. The bacteria were labeled by placing them on protein-A gold 5 nm diluted 1:70 in 1% FSG for 30 minutes. Washing were performed five times with 120 mM HEPES then five times with distilled water for 1 minute each time. Staining were performed by briefly passing a droplet of 1% uranyl acetate (UA) to remove excess water, before leaving it on a new drop of UA for 1 minute, then grids were washed with distilled water twice for 1 minute each. The grids were placed on droplet with 2% methyl cellulose for 1 minute as a pick-up solution to preserve morphology of bacteria, or directly dried. The grids were then stored in a grid storing box.

Due to unspecific binding of gold particles to the negative control (*F.n.n. clpB* mutant), and to verify that the binding of the antibodies to the *F.n.n.* mutants is truly due to the presence of the antigen and not random, a negative controls were added for each mutant. The negative controls were created by omitting the primary antibody, which should then ensure that no second antibody binding should occur, and therefore no labelling with gold particles. The following improved protocol were used:

Before incubating on bacterial solution, grids were washed with distilled water for 3-5 minutes to reduce charge of the grid. Washing steps before FSG incubation was reduced from tree to two. Concentration of both primary and secondary was further diluted from 1:200 to 1:250 to reduce unspecific binding. All washing steps with HEPES were reduced from five to three times. Both before incubation with secondary antibody and gold, an extra step of incubation on FSG were added to improve blocking of unspecific binding sites (as done by Brudal et. al (137)).

Extra immunogold controls attempt

An attempt was made to include a positive control for the immunogold labeling protocol, since no distinct pili structures were visible on *F.n.n.* Both mutants and wild type *Neisseria gonorrhoeae* (*Ng*) bacteria were utilized (see Table 11). *Ng* bacteria were grown and kindly donated by Chris Hadjineophytou from The Koomey team at the Department of Bioscience, (University of Oslo). The controls were prepared by the same improved immunogold protocol, and a negative control included where incubation with the primary antibody were omitted.

Table 11 Neisseria gonorrhoeae used for positive immunogold control.

Neisseria gonorrhoeae	Pili	Reference
Wild type N400	Naturally present, no tag	(162)
Mutant KS538	Induced by IPTG, 6His tagged *pilE*	(162)

2.2.8 Transmission electron microscopy

The grids prepared by utilizing the immunogold labelling or negative stain protocols were viewed in a JEOL 1400PLUS transmission electron microscope.

2.2.9 Pilot trial before flow cytometry

A pilot trial was performed to evaluate the method before utilizing the protocol for the flow cytometry. *Francisella* mutants *F.n.n. ΔpilD pilA1:his, F.n.n. pilA1:his* and *F.n.n. clpB* were used. THE™ His-tag mouse monoclonal antibody [iFluor488] (Genscript) and 4',6-diamidino-2-phenylindole (DAPI) were used (Table 12). Concertation of DAPI used was approximately 2.4 mg/mL. DAPI is capable of coloring dsDNA by binding to adenine:thymine rich regions, staining the nucleus of cells blue. Microscope filters (Table 13) and details about fluorescent compounds used (Table 12) can be found below.

Table 12 Fluorophore used for pilot trial before flow cytometry.

Fluorescent compound	Excitation (max)	Emission (max)	Filter used
DAPI	350 nm	470 nm	UV-2A
THE™ His-tag mouse monoclonal antibody [iFluor488] (Genscript)	490 nm	525 nm	B-2A

Table 13 Nikon cube 1 Filter specifications (Nikon®).

Filter	Excitation filter wavelengths	Dichromic mirror cut-on wavelength	Barrier filter wavelength	Detectable florescence emission regions
B-2A	450-490 nm	500 nm	515 nm	Green, yellow, red
UV-2A	330-380 nm	400 nm	420 nm	Red, green, blue

Cover slip were coated with poly-L-lysin (Sigma-Aldrich) diluted 1:2 in distilled water for 30 minutes at room temperature. Slips were washed with distilled water a total of three times by placing it coated side down onto droplet. Bacteria were harvested by scooping bacteria from a plate, using a sterile loop. Bacteria were transferred to an Eppendorf tube with 0.5-1.0 mL Eugon broth or 60 mM HEPES and dispersed, using a vortex mixer. Bacteria were fixed by adding paraformaldehyde to the bacterial mixture to a total of 2% and incubated for 10 minutes. 100-200 µL bacterial solution were added to coated side of slip and incubated for 30 minutes, covered by a lid to avoid drying. Slips were washed with PBS a total of three times.

Bacteria were labeled by placing slip coated side down on drop of THE™ His Tag Antibody [iFluor 488] (mAb, Mouse) diluted in PBS mixed with 0.5% bovine serum albumin (BSA), and incubated for 60 minutes, covered (see Table 14 for dilutions). BSA is used as a blocking buffer to avoid unspecific binding of the antibody. Samples that were not to be tagged with antibody, were left for 60 minutes on PBS mixed with 0.5% BSA only. Slips were then washed with PBS a total of three times. Bacteria were stained by placing slide face down on droplet of DAPI diluted in PBS with an approximately concentration of 0.25 µg/mL, for 20 minutes. Slips then were washed with PBS a total of two times. Mounting was done by placing slips on droplet of Mowiol (Sigma-Aldrich) in DABco (Sigma-Aldrich) on microscope glass slide.

Slides were examined by using a fluorescent microscope (Nikon Eclipse TE300). DAPI stain were viewed using UV light and fluorescent from the conjugated iFluor 488 antibody were viewed using the B-2A filter in Nikon microscope.

Table 14 Treatment regime of Francisella mutants used. A Nikon microscope with the Nikon cube 1 were utilized.

Anti-His mAb [iFluor 488] dilution	DAPI	Nikon cube 1 lens*
No	No	None
1:50	No	B-2A
1:50	Yes	B-2A, UV-2A
1:100	No	B-2A
1:100	Yes	B-2A, UV-2A
1:500	No	B-2A
1:500	Yes	B-2A, UV-2A
1:1000	No	B-2A
1:1000	Yes	B-2A, UV-2A

*See Table 12 for filter specification.

2.2.10 Flow cytometry

Flow cytometry is a method for both counting and sorting cells by utilizing antibodies conjugated to a fluorescent compound. This method can also be used to measure chemical and physical qualities. The technique is based upon the scattering of light by uniformly passing cells through a laser beam, like pearls on a string. In addition to the light being scattered by cells individually passing the laser beam, the fluorophore attached to the antibody emits light with a wavelength characteristic for the fluorophore (see Table 15 for fluorescent compounds used). The two main ways which light scattering are measured is by forward (FSC) and side scattering (SSC). FSC can estimate particle size, where a higher degree of scattering is linked to a larger sized particle. SSC is light scattered by physical characteristics on the particle and can give an indication about particle complexity. Density plots can be constructed based upon measurement parameters of interest. Dense regions of signals will appear as separate populations, based upon the sample components. The population of interest can then be further analyzed by gating the area of interest. Gates are areas marked by the user, which can be displayed in a secondary graph and give information only based on the selected population of interest. In this thesis, the information of interest was the fluorescence emitted by the anti-his antibody and the propidium iodide is used to assess the presence of live or dead bacteria.

Table 15 Fluorescent compounds used in flow cytometry.

Fluorescent compound	Excitation (max)	Emission (max) /color	Filter used
iFluor 488	490 nm	525 nm/green	488-526 nm
Propidium iodide	535 nm	617 nm/red	355-620 nm

A flow cytometer is also capable of sorting. In this thesis, sorting was based upon the attachment of an anti-his antibody attached to an iFluor 488. *F.n.n.* were sorted by instructing the flow cytometry to transfer any bacteria marked with the antibody, which is characterized by the light emitted by the fluorophore, as well as not being labeled with PI, to a clean tube (163).

The following improved protocol was utilized for flow cytometry.

The bacteria were harvested by scooping bacteria from a plate, using a sterile loop. Bacteria were transferred to an Eppendorf tube with 0.5-1.0 mL Eugon broth and dispersed, using a vortex mixer. Bacteria were fixed by adding paraformaldehyde to a total of 2% and incubated for 10 minutes. The bacteria were spun down using a tabletop centrifuge at 12.000 rpm for 4 minutes and supernatant removed. Washing were performed by re-suspending the bacteria in sterile filtered PBS, spinning for 4 minutes, and removing supernatant. Washing were repeated twice. The bacterial pellet was re-suspended in 100 µL of antibody diluted 1:500 in PBS mixed with 0.5% BSA and incubated for 60 minutes. Samples that were not to be tagged with antibody were re-suspended in PBS mixed with 0.5% BSA only. Bacteria were spun down, and supernatant removed. Washing were performed twice with sterile filtered PBS as explained earlier. 100-500 µL of PBS were added to each sample, and bacterial pellet re-suspended. 15-17 µL of 2 mg/mL propidium iodide (PI) were added to 500 µL bacterial solution and incubated in darkness (due to PI being light sensitive) on ice for 1 minute before analysis in flow cytometry. Presence of *Francisella* bacteria were chosen based upon the forward scattering of the *F.n.n. clpB* control mutant which were neither PI stained, nor antibody labeled. The occurrence of PI stained and his-positive antibody labeled bacteria were made upon assessing the presence of emitted fluorescence from these compounds. His-tagging were exclusive for bacteria with his-tagged pilin genes, as *F.n.n. clpB* mutant did not show any emissions when treated with fluorescence labeled antibody. Sorting of his-tagged bacteria were done by utilizing the sorting function in the flow cytometry. Sorted bacteria were cultured on ECA plates and used for further analysis.

2.2.11 Atomic force microscopy

Atomic force microscopy (AFM) is a scanning probe microscopy technique which utilizes raster scanning (rectangular image pattern where each pixel is acquired via sequential contact/near-contact with the sample surface). The main elements of AFM are the cantilever, a laser and photodiode and a movable stage. The laser is aimed at the cantilever and is reflected towards the photodiode. The sample is moved right to left by the movable stage, in which the cantilever follows the sample surface. The cantilever is displaced as it moves across the surface sample, which in turn affects the laser light reflected from its surface, in which the signals create a 3D image of the sample surface. Even though AFM is also able to perform force measurements, in this thesis it was only used for topological analysis of *F.n.n.* to determine the possible presence of a type IV pili system (164).

Epoxy were mixed and added to glass slides by using a pipette tip. A square piece of mica (sheet silicate mineral used to create flat and thin sample surfaces in AFM) were placed on top of the epoxy, pressed down, and left to dry. The mica was smoothed out and negatively charged by placing a piece of crystal tape on top and dragging of the uppermost surface of the mica a total of six times. 20 µL of bacterial solution were placed in a PCR tube and 5 µL of Tris/$MgCl_2$ and 25 µL of milli-Q water added to suspension. 10 µL of bacteria suspension were added on top of the mica and left to dry for 10-15 minutes. The mica was then cleaned by pipetting 100 µL of milli-Q water over its surface at an angle a total of ten times. The surface was then dried by utilizing nitrogen gass to carefully blow off residual water. Samples were then viewed in a NanoWizard 2004 version 1.1 (JPK BioAFM) atomic force microscope by tapping method using a cantilever (Z-range 5,85µm, target amplitude 2V). AFM pictures were taken with aid from Ida Kristin Hegna at the Department of Pharmacy, University of Oslo.

2.2.12 PCR verification of *Francisella*

Bacterial strains sorted with the flow cytometry could be exposed to contamination due to the possible non-sterile environments from transfer from flow cytometry sorting to new ECA plates containing kanamycin. After growth (6-7 days), colonies that showed similar morphology and color as *F.n.n.* were transferred to new ECA plates with kanamycin. To verify the presence of *F.n.n.* amongst these sorted bacteria, PCR with specific *Francisella* primers were performed (Table 16). PCR reactions were prepared by pipetting the following reagents and amounts mentioned in Table 17. See Table 18 for PCR program.

Table 16 Primers used for PCR verification of F.n.n.

Target	Forward 5'→3'	Reverse 5'→3'	Amplicon length (bp)	Ref.
***Francisella noatunensis* subsp. *noatunensis* (Diagnostic)**	TGAGTTGGTAACCATTGATTGTACATAGT	CGAGTACCTGGTGGGAGAAAGA	97	(165)
F.n.n_iglC_O	GGTGGTGAACCATATGGCGC	TAAAGTCCCATTCCGAGCCTT	2271 in mutant, 2837 in wt	(127)
F.n.n_iglC_C1	TAAAGTCCCATTCCGAGCCTT	GTGTATGTTGCAGAATTTGATCA	2268 in mutant, 2864 in wt	(127)

Table 17 PCR reaction mixture content for the F.n.n. verification.

Reagent	Amount (µL)
dH_2O	Up to 25.0
10X optimized DyNAzyme buffer	2.5
10 mM DNTPs	0.5
Forward primer (100 µM)	1.0
Reverse primer (100 µM)	1.0
Template DNA/bacterial solution	3.0
DyNAzyme II DNA polymerase (2U/µL)	0.5

Table 18 PCR program used for F.n.n. verification in a PCR instrument (Applied Biosystems® 2720 Thermal cycler).

Step	Step	C°	Time	Cycles
Initial denaturation	1	94	5 minutes	1
Denaturation	1	94	30 seconds	45
Annealing	2	60	10 seconds	
Extension	3	72	40 s/kb	
Final extension	1	72	7 minutes	1
Cooling	1	4	Hold	1

2.2.13 Bacterial DNA extraction

For the purpose of PCR, DNA from *F.n.n.* were extracted from bacterial cultures on ECA plates using the DNA mini kit from QIAMP (see Appendix 2).

2.2.14 *Escherichia coli* control

Due to unsatisfactory results from PCR with *F.n.n.* mutants, *Escherichia coli* (*E. coli*) mutants were used as a control to ensure that the PCR protocol works optimally. *E. coli* mutants (Table 19) were streaked out on ECA plates containing kanamycin and incubated at 37°C for 1-2 days. Plasmids from both mutants were obtained using the plasmid extraction kit from E.Z.N.A.® (see Appendix 3). Primers for m*Cherry* plasmid and Fnn_compl_tr_iglC were used for both plasmids (Table 20). PCR product were run on an agarose gel and products only retrieved for plasmids with its belonging primers. To verify size of extracted plasmids, a restriction enzyme reaction with restriction enzyme SpeI (New England biolabs) were used. See Table 21 for restriction enzyme reaction components. Mixture were incubated in a digital dry bath (Labnet) at 37°C for 1 hour and products ran on an agarose gel.

Table 19 E. coli mutants used as controls

***Escherichia coli* mutants**	**Plasmid**	**Plasmid size (bp)**	**Reference**
***E. coli* mCherry**	- pKK289Km/m*Cherry*	3.300	(166)
***E. coli* DH5αpir**	- pKK289Km/*iglC*	~5.000	(127)

Table 20 Primer used for PCR of control (E. coli).

Target	**Forward 5'→3'**	**Reverse 5'→3'**	**Amplicon length (bp)**	**Ref.**
m*Cherry* plasmid	TACACATATGGTG AGCAAGGGCGAGG	CACCAGACAAGTTG GTAATGG	778	(166)
Fnn_compl_tr_iglC	TCTGCACATATG*A TTATGAACGAGAT GATAACAAGACAG	CTGAGAATTC**CTA TACAGCTGCTATGTA CCCTATTCT	635	(127)

*NdeI restriction cutting site. **EcoRI restriction cutting site

Table 21 Restriction reaction reagents used for E. coli controls.

Reagent	Amount (µL)
Plasmid	5.0
SpeI restriction enzyme (NEB)	0.5
Restriction enzyme buffer (CutSmart/NEBuffer™2, NEB)	2.0
dH_2O	12.5

2.2.15 Plasmid extraction

Plasmid extraction from *Escherichia coli* mutants were performed by utilizing the plasmid DNA mini kit I from E.Z.N.A.® (see Appendix 3).

2.2.16 Agarose gel

1% agarose gel was made by adding 0.5 g agarose powder to 50 mL TAE 1x buffer (pH 8.3). The solution was heated until the powder was completely dissolved. After cooling down to approximately 60 °C, 5 µL of GelRed® nucleic acid stain (Biotium) was added to solution and mixed. Solution was then poured into gel casting tray and a comb with the needed amount of wells was added. The gel was left to dry in a fume hood. When dry, the gel was placed into a gel tank filled with TAE 1x buffer. When completely submerged under buffer, the comb was removed. To each well, 8 µL of PCR product mixed with 2 µL of loading buffer was added. 6-10 µL of 100bp or 1kb ready-to-use DNA ladder (Thermo Fisher Scientific) was added to a separate well. The gel was run at 80-90 V for 60-90 minutes and inspected using a gel doc (Bio-Rad).

2.2.17 Sequencing

Due to unsatisfactory results from PCR samples of *F.n.n.* with several primers, sequencing were performed to verify the presence of *F.n.n.* Both extracted DNA and live bacteria were used for PCR with 16S rRNA primers (Table 22). Samples with approximately 24 ng/µL PCR product and forward and/or reverse primer were prepared and shipped to Eurofins for GATC services-LightRun tube sequencing (167). Even though the sequencing results from *F.n.n. pilA1:his* came back as *Francisella,* these bacterial colonies had been exposed to a contaminant and were lost from further analysis. Sequencing results from mutants *F.n.n. ΔpilD pilE6:his, F.n.n. ΔpilD pilA1:his* and *F.n.n. pilA6:his* also indicated that these were *Francisella* and were used for further analysis (see Appendix 5)

Table 22 16S rRNA primer used for sequencing of F.n.n.

Gene	Forward 5'→3'	Reverse 5'→3'	Amplicon size
16S rRNA	ATGGCTGTCGTCAGCT	ACGGGCGGTGTGTAC	350

3 Results

The aim of the experiments in this thesis is linked to vaccine development against fish diseases that are known to be common in aquaculture and are responsible for devastating economical loss globally.

The vaccine trial was conducted in the auspices of the University of Oslo, at the Department of Pharmacy and Norwegian University of Life Sciences (VESO-3566, 2016). The vaccine consisted of MVs harvested from *P. salmonis* strain LF-89. Atlantic salmon were immunized with MVs in the hope of preventing the onset of piscirickettsiosis from co-habitation with *P. salmonis*. Tissue from this trial were analyzed to assess any possible protective aspects present. Difference in protection by immunization with MV based vaccines were examined by utilizing two vaccine groups, in which the concentration of MV and addition of adjuvant varied. Histological analysis were also conducted to evaluate the presence of disease markers as granulomas. Evaluating the effect seen from immunization with MVs may aid in further vaccine development. This is possible by assessing the difference in both immune gene expression and disease markers between immunized fish and control, and between the two immunized groups.

Presence of a Tfp system was evaluated in *F.n.n.* utilizing flow cytometry, immunogold labeling and examination in TEM, and AFM. The foundation of the experiments stems from the discovery of several intact Tfp genes in *F.n.n.* by Dr. Espen Brudal (137), indicating a possible functionality and/or presence of pili (see section 1.6.2, Figure 3). Characterization of an intact Tfp system may aid in further understanding of the virulence mechanisms of *Francisella* as well as contributing to vaccine development against francisellosis.

3.1 Evaluation of MV-based *P. salmonis* vaccine effect on Atlantic salmon

Experiments assessing the immunological and histological responses after immunization with a vaccine based on MVs from *P. salmonis* were performed. This was achieved by utilizing tissue from both non-infected and *P. salmonis* infected Atlantic salmon (kidney, spleen, and liver). The non-infected fish (0 weeks post challenge (WPC)) were harvested 7 weeks after immunization and before co-habitant infection with *P. salmonis.* Infected fish were harvested 4 WPC with *P. salmonis* (11 weeks after immunization).

Six vaccine trial groups were used in the vaccine trial. Group 6 consist of the shedders, which are fish injected with *P. salmonis* and as the name suggest, is used to spread *P. salmonis* by co-habitant infection. Group 3 and 4 are both non-immunized fish, in which the difference is the injection of saline in group 3 (control group). This group is chosen as the control rather than group 4 (untreated, non-immunized), as part of a sham treatment. Group 1 and 5 are the MV-based vaccine groups. Group 1 contains 40 µg MV and adjuvant (Montanide ISA 780 VG), while group 5 only contains 100 µg MV. Both different concentration of MV administered and the addition of an adjuvant are used to assess any difference in protection in relation to these parameters. Group 2 are administered adjuvant only and are used to assess how the adjuvant on its own affects the immunological response and if this relates to any difference between the MV based vaccine groups. The aim of this experiment was to identify any significant immunological differences between fish from vaccinated and control groups, as vaccine did not yield the desired protection.

3.1.1 Immunological response of vaccine trial

As part of an end evaluation of a vaccine trial based on MVs harvested from *P. salmonis,* a quantitative analysis was performed by evaluating the expression of immune related genes. The vaccine trial was performed in Atlantic salmon, in which MV immunized fish were infected 7 weeks post vaccination with *P. salmonis* by co-habitation. No signs of protection were seen, when assessing the cumulative mortality in any of the MV immunized group compared to the control (see cumulative mortality section 2.1 Figure 5). However, it was of interest to evaluate if immunization had any possible protective effect by assessing the expression of a set of immune genes. Examining the genetic expression of immune related genes between differently immunized groups, may help in further development of MV based vaccines, concerning both concentration of injected MV as well as the addition of adjuvant.

Figure 6 A, C and E shows the relative expression levels of selected immune genes in the spleen, liver, and kidney tissue (respectively) at 0 WPC from Atlantic salmon that have been vaccinated against, but not infected by *P. salmonis*. Figure 6 B, D and F shows gene expression in spleen, liver, and kidney (respectively) at 4 WPC and represents immune expression from Atlantic salmon that have been vaccinated against and infected by *P. salmonis* by co-habitation. A result is declared as statistically significant when $P<0.05$. The following sections reviews the statistically significant results, unless other is stated.

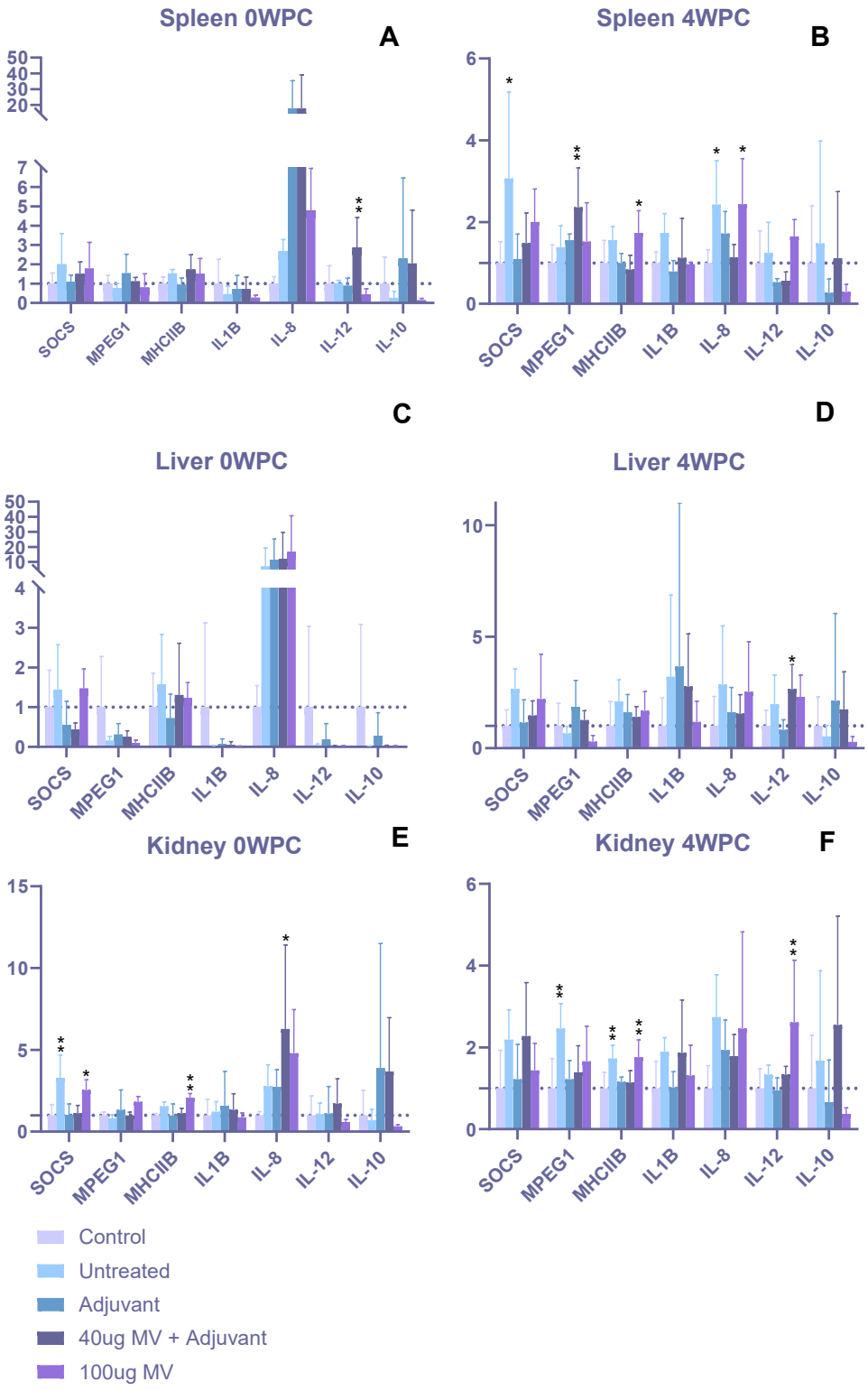


*Figure 6 Relative expression of immune genes in kidney, spleen and liver from Atlantic salmon after a 7 week immunization period with P. salmonis derived MVs (time 0) and 4 weeks after challenge with P. salmonis 0 WPC: Immunized, non-infected fish. 4 WPC: immunized, P. salmonis infected fish. Two MV- based vaccine groups are used to assess protective differences when injection different concentration of MVs and the effect of adjuvant addition. Bars represents mean values with whiskers representing standard deviation. Values that are statistically significantly different to the control are denoted by asterisk in the following pattern: *p<0.05, **p<0.01, ***p<0.001. Significance were determined by the use of one-way ANOVA in Graphpad Prism 8. Control is equalized to 1, indicated by dotted line.*

In the spleen at 0 WPC, a significant induction of IL-12 is seen within the 40 µg MV + adjuvant group with a 2.87 fold increase compared to the control. Although not statistically significant, an apparent induction of IL-8 was observed in the adjuvant and 40 µg MV + adjuvant groups (17.64 and 17.65 fold increase respectively). This was also true in the liver at 0 WPC for all groups compared to the control (fold increase: untreated (6.99), adjuvant (11.37), 40 µg MV + adjuvant (12.16) and 100 µg MV (16.67)). However, no statistically significant changes were seen within the liver at 0 WPC.

In kidney at 0 WPC, statistically significant differences were seen for SOCS, MHCIIB and IL-8. A 2.54 and 2.07 fold increase was seen in the 100 µg MV group for SOCS and MHCIIB, respectively. A statistically significant increase of IL-8 expression was seen within the 40 µg MV + adjuvant group, though to a lesser extent than in the spleen and liver with a fold change of only 6.28. In the untreated group, an induction in SOCS expression were seen, with a fold increase of 3.30 compared to the control.

In the spleen at 4 WPC, an increased gene expression is observed for several genes across multiple groups. For the 40µg MV + adjuvant group, a 2.37 fold increase is seen for MPEG1. For the 100 µg MV group a 1.73 and 2.43 fold increase is seen for MHCIIB and IL-8, respectively. However, as seen in the kidney at 0 WPC, an unexpected statistically significant fold increase of SOCS and IL-8 is seen in the untreated group (3.07 and 2.43 fold increase respectively) in the spleen. As for spleen at 0 WPC, only one gene is statistically significantly up-regulated within the liver at 4 WPC. The expression of IL-12 within the 40µg MV + adjuvant group showed a 2.65 fold increase compared to the control. In the kidney at 4 WPC, expression of several genes was statistically significantly increased. Within the 100 µg MV group, a 1.76 and 2.62 fold increase were seen for MHCIIB and IL-12 respectively. However, as seen in the kidney at 0 WPC and spleen at 4 WPC, a fold increase in expression of genes in the untreated group were seen also in the kidney at 4 WPC. This time, a statistically significant fold increase of 2.46 and 1.72 was seen for MPEG1 and MHCIIB, respectively.

3.1.2 Histological examination of vaccine vs. control group kidney and spleen tissue

To assess the possible protective effect after immunization with MVs, histological analysis were performed on tissue from both non-infected fish and *P. salmonis* infected fish, to determine any difference in disease progression. Three biological samples from kidney and spleen were used, at two different time points. Only the immunized groups (40 µg MV+ adjuvant and 100 µg) and control group (saline) were examined. The aim of conducting a histological examination was to evaluate the presence of granulomas, which are known disease markers for piscirikettsiosis.

Both toluidine blue (Figure 7B) and PAS staining (Figure 7A) were attempted. Structures were easier distinguishable when using PAS staining (Figure 7A) and were therefore used as dye of choice for all following tissue samples. Tissue of fish at 0 WPC is generally referred to as non-infected or "healthy", because its health conditions may not solely be determined by whether it is infected or not. Tissue from 4 WPC are in general referred to as infected.

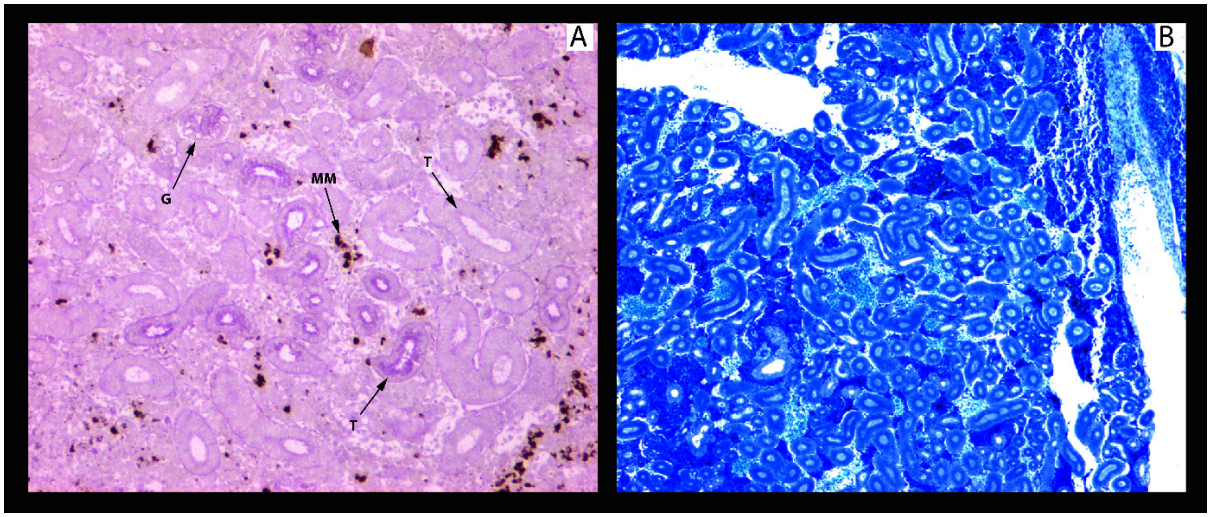


Figure 7 shows the difference in staining quality between PAS (A) and toluidine blue staining (B) in kidney tissue. A: several structures are visible when using PAS staining: glomerulus (G), tubules (T) and melanomacrophages (MM). B: The difference in staining is most evident when examining the presence of MM, in which no distinct MM are visible when using toluidine blue staining. 10x magnification is used.

Histological examination showed no apparent presence of granuloma in the spleen of any of the groups investigated, at neither 0 WPC nor 4 WPC. However, a distinction could be seen between the non-infected and infected fish (0 and 4 WPC, respectively). This difference is particularly noticeable in the spleen of the 40 µg MV + adjuvant group, in which tissue formation appear more disturbed with fewer separately distinguishable cells and a reduced overall "smooth" appearance (Figure 8 C and D). Several vacuoles were present in spleen of *P. salmonis* infected fish in the control (saline) group (Figure 8A). Tissue from infected fish in the 100 µg MV group showed several cells with enlarged nuclei, which is seen as darker purple spots (Figure 8E) compared to the non-infected fish in this group (Figure 8F). In general, an increased amount of melanomacrophages was seen in tissue of infected fish compared to non-infected fish (red/brown spots) in the spleen.

No granulomas were present in the kidney tissue of infected or non-infected fish. As for the spleen, tissue from infected Atlantic salmon kidney showed a more disturbed apperance, in addition to several enlarged nuclei. The amount of melanomarophages in kidney were abundant in both non-infected and infected kidney tissue, and can on its own not be linked to disease progression (Figure 9).

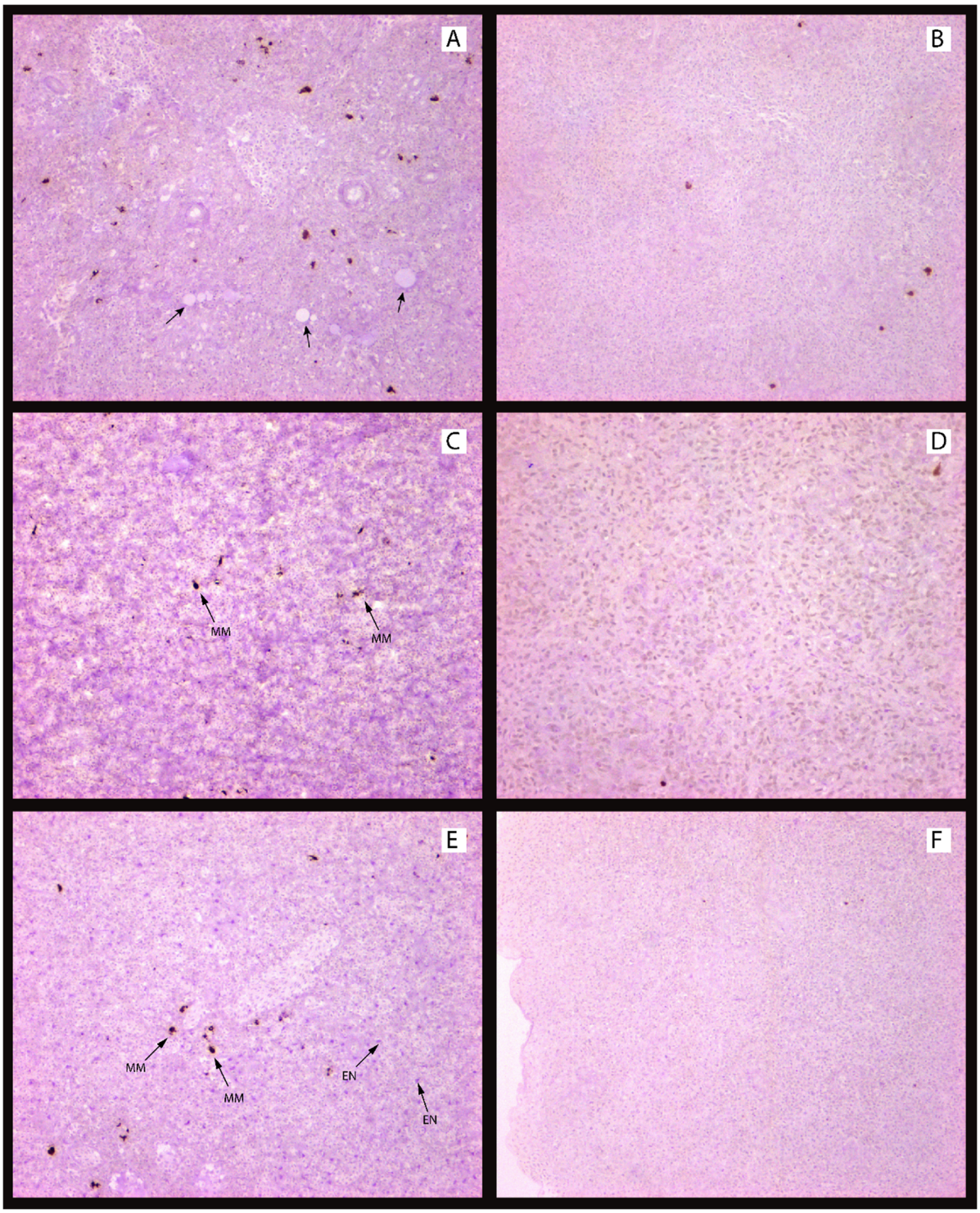


Figure 8 Histology samples from Atlantic salmon spleen. A-B: control (saline injected) vaccine trial group of fish from 4 and 0 WPC (A and B respectively). Presence of vacuoles is seen in A (arrows) as well as melanomarophages (dark red/brown spots). C-D: Fish immunized with 40 µg MV + adjuvant from 4 and 0 WPC (C and D respectively). E-F: Fish immunized with 100 µg MV from 4 and 0 WPC (E and F respectively). Presence of enlarged nuclei (EN) is seen in E as dark purple spots. All pictures except D are taken in 10x magnification, D is 20x. Hematoxylin and Periodic acid Schiff's staining is used.

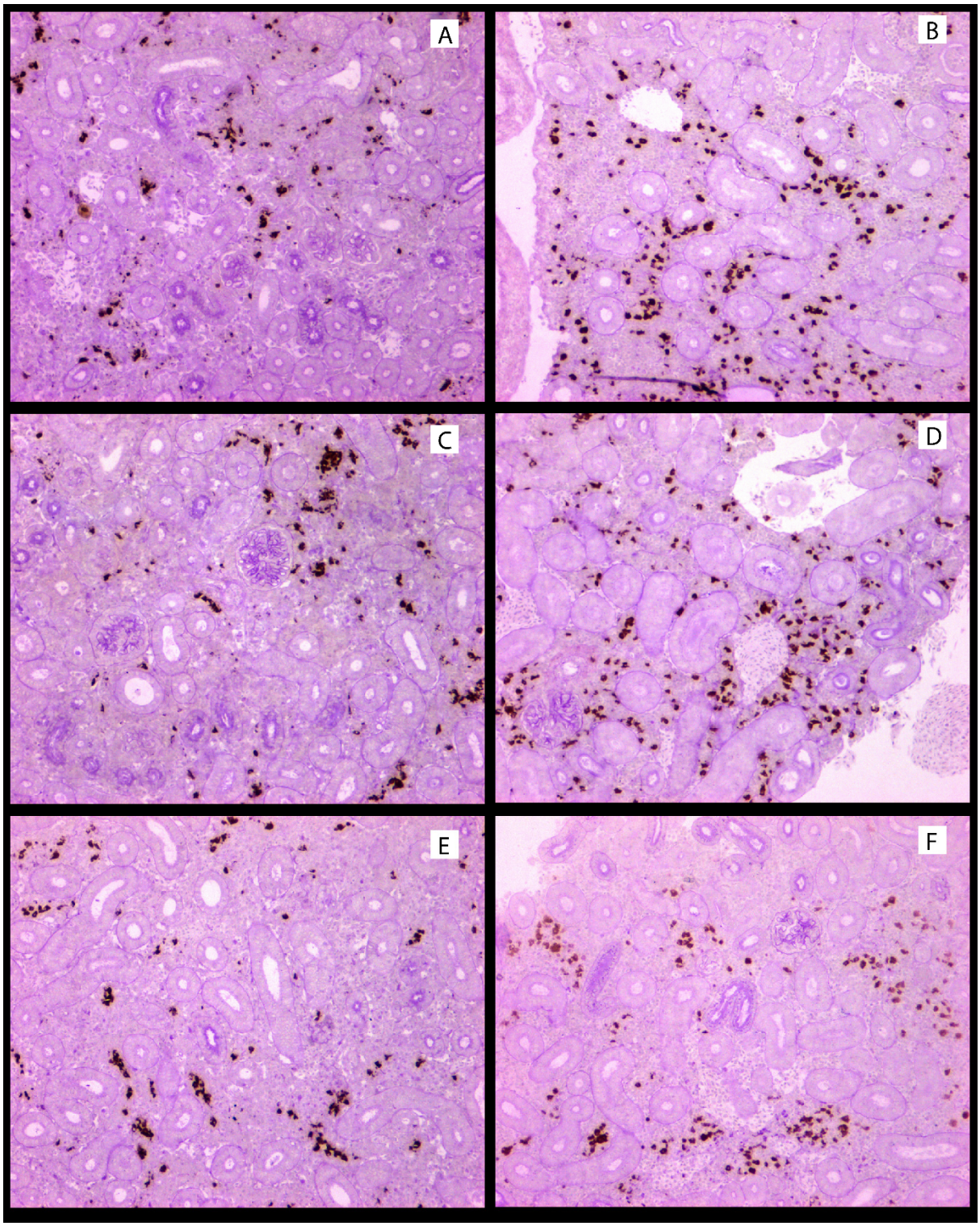


Figure 9 Histology samples from Atlantic salmon kidney. A-B: control (saline injected) vaccine trial group of fish from 4 and 0 WPC (A and B respectively). Some tubules appear darker stained with PAS staining. C-D: 40 µg MV + adjuvant group from 4 and 0 WPC (C and D respectively). Some tubules appear darker stained with PAS staining. E-F: 100 µg MV group from 4 and 0 WPC (E and F respectively). Darker red/brown spots are melanomacrophages. All pictures: 10x magnification, hematoxylin and Periodic acid Schiff's staining.

Though no granulomas were seen in neither kidney nor spleen, other characteristics of inflammation or negative response of infection were seen. In tissue of fish vaccinated with 40 µg MV + adjuvant, a possible abnormality or artifact was seen within blood vessels of infected fish (Figure 10A, B and C). Figure 10A, B and C are sections from the same biological sample and are separated by 12-50 µm and the abnormality seen within all pictures are most likely part of the same structure. However, structures seen in Figure 10 C may also indicate a possible thrombi or inflamation within the vessel. In kidney tissue from infected fish from the control group, a patch of less organized cells and what seems to be cytoplasmic vacuolization were seen (Figure 10E). However, more clearly distinguishable vacuoles were seen in spleen of infected fish of the control group (Figure 8A). In kidney of infected fish in the 100 µg MV group, a larger area of unorganized cell distribution were seen (Figure 10F). The tissue shows clear signs of a general tissue-reaction/degeneration which one may sepculate culd lead to the formation of a granuloma.

Another noteworthy structure from the histological examination is the distinguishable features of red and white pulpa seen within the spleen in Figure 10D. Red pulp is comprised of blood cells and connective tissue while white pulp is mainly lymphoid-like tissue containing ellipsoids, leukocytes and melanomacrophages Unlike the spleen of mammals, fish do not possess distinct compartmentalization of red and white pulpa. However, a local collection can be seen here, in which the melanomacrophages are confined within the red pulpa, surrounding the white pulpa. The reason why red pulpa is less stained, is because blood cells is less sufficiently stained with PAS-stain compared to tissue, and which makes white pulpa more purple.

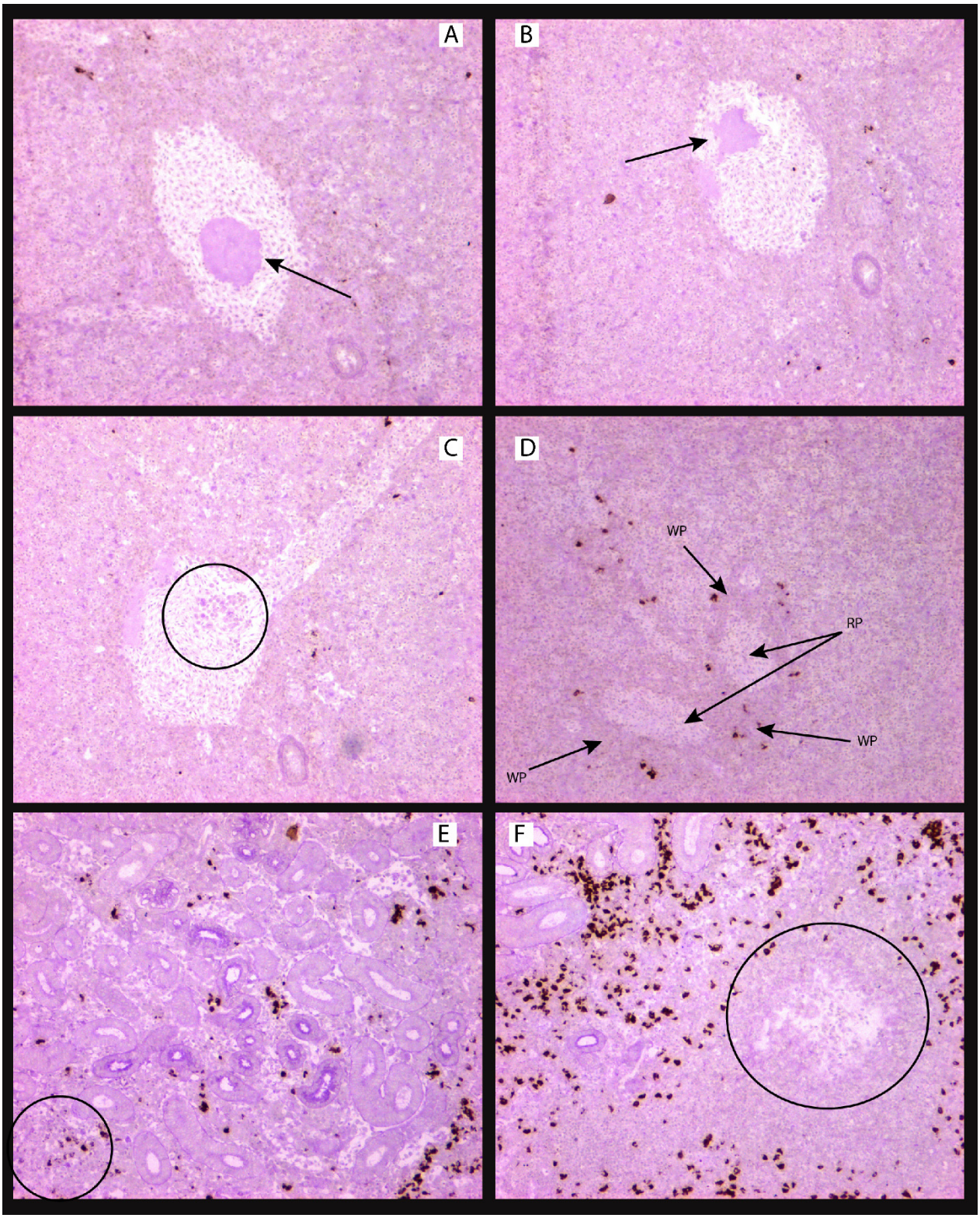


Figure 10 shows an excerpt of possible disease markers related to infection with P. salmonis in both kidney and spleen tissue from Atlantic salmon at 4 WPC. A-B: 40 µg MV + adjuvant group (4 WPC). Shows presence of a possible abnormality or artifact in a blood vessel of the spleen. C: 40 µg MV + adjuvant group (4 WPC), possible thrombi or inflammation in a blood vessel of the spleen. Section seen in C is from the same sample as sections in A and B and may be part of the same abnormality or artifact. D: Control group (4 WPC), shows a more distinct compartmentalization of red (RP) and white (WP) pulp of the spleen. E: Control group (4 WPC), shows most likely presence of vacuolar degeneration. F: shows local tissue damage in 100 µg MV immunized fish (4 WPC). All pictures: 10x magnification, hematoxylin and Periodic acid Schiff's staining.

3.2 Evaluation of Tfp presence in *Francisella noatunensis* subsp*. noatunensis*

Experiments were performed by utilizing mutant *Francisella noatunensis* subsp*. noatunensis,* constructed by Espen Brudal. The aim of this analysis was to perform immunogold on both mutant and wt *F.n.n.* to evaluate the presence of type IV pili using a TEM and AFM. The possible presence of Tfp expression on the surface of *F.n.n* was tried using this method by the use of 6-histidine tagged pili proteins together with antibodies that recognize the 6-His tag. Both *F.n.n.* mutants containing 6-his tagged pili proteins only and *F.n.n.* mutants containing a disrupted PilD Tfp subcomponent and 6-his tagged pili proteins were examined. The mutants containing a disrupted PilD component are denoted *ΔpilD*. Pili proteins his-tagged are *pilA1, pilE2, pilE4, pilE5* and *pilE6* and are denoted *pilA1:his* etc.

3.2.1 *F.n.n.* showed no presence of pili

For optimal trustable results, immunogold electron microscopy experiment consisted of several phases of trial and error. The possible presence of Tfp expression on the surface of *F.n.n.* was tried using this method by the use of 6-histidine tagged pili proteins together with antibodies that recognize the 6-His tag. In general, *F.n.n*. are fragile and easily burst when treated with the different reagents which makes transmission electron microscopy of intact bacteria challenging. The first protocol (see section 2.2.7) gave images of acceptable quality of *F.n.n.* itself (Figure 11). However, due to some unspecific binding of gold particles to the control *F.n.n.* pKK:*gfp* strain (Figure 11A), there were no significant difference in labeling between the control and the *F.n.n.* mutants (*F.n.n. pilA1:his* (Figure 11B) and *F.n.n. pilD pilA1:his* (Figure 11C)). Validation of specific binding towards his-tagged pili components in the outer membrane of *Francisella* could therefore not be made based upon the acquired results.

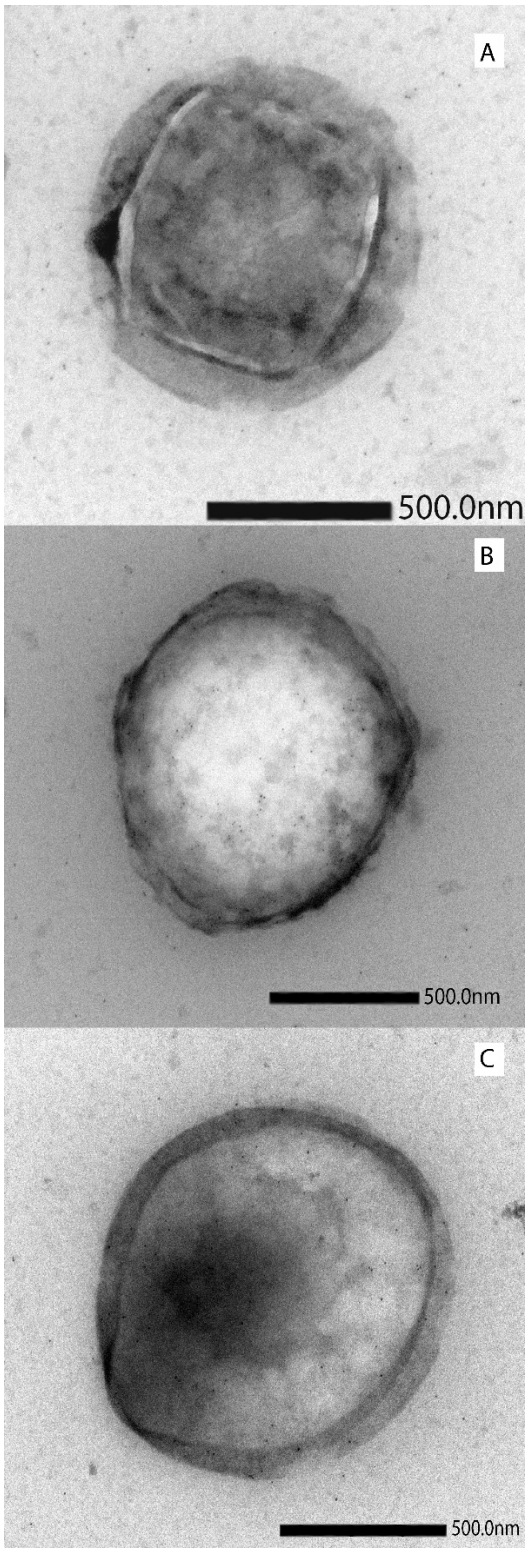
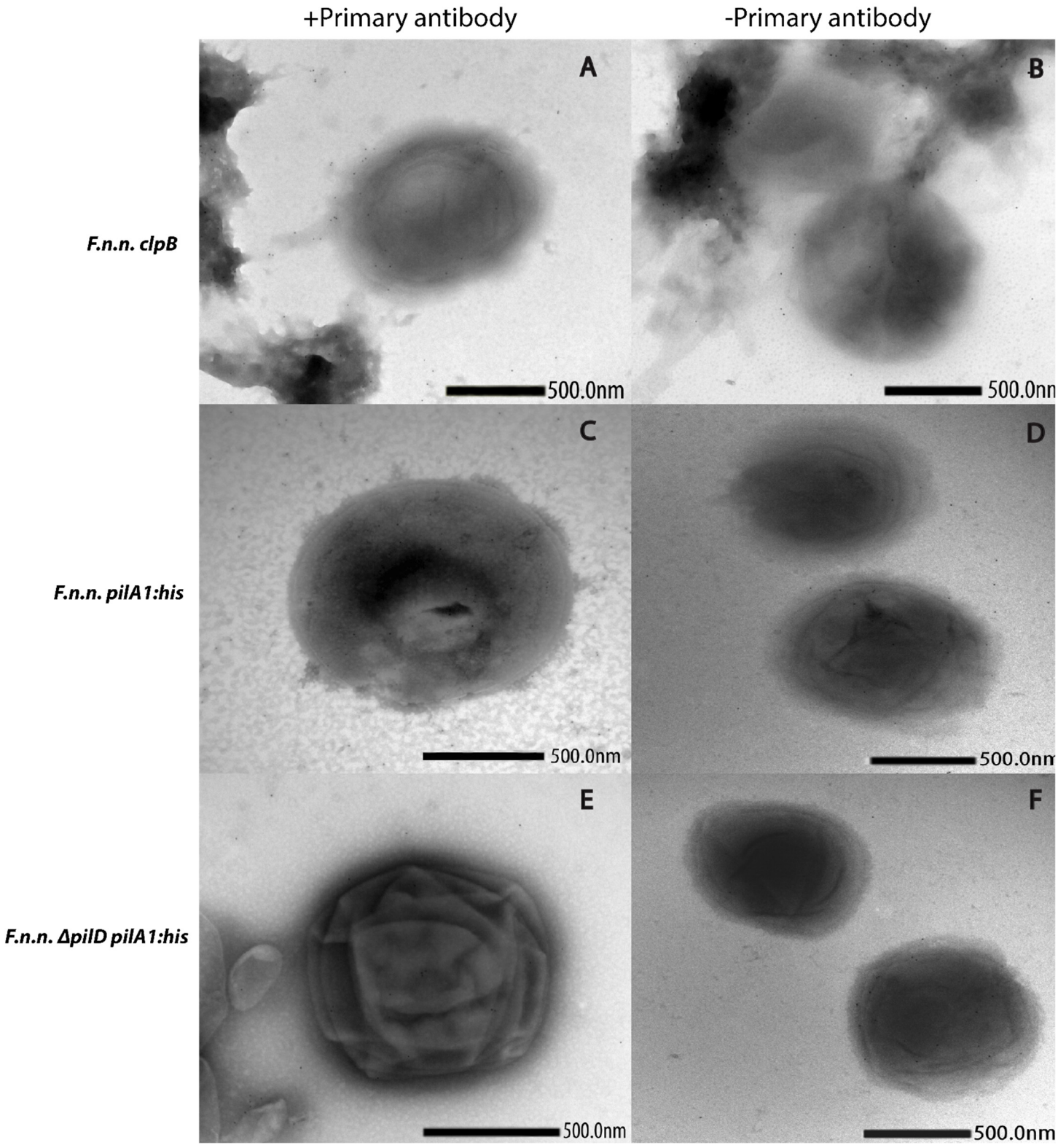


Figure 11 pictures obtained from the first immunogold trial experiment. Compares gold labeling between control (without pili tag) and pili-tagged samples. A: F.n.n. pKK:gfp (control). B: F.n.n. PilA1:6his. C: F.n.n. ΔpilD pilA1:his.

To further optimize and validate the results, changes were made to the protocol. The main changes included an increased dilution of both primary and secondary antibodies, as well as omitting the primary antibody. By further increasing the antibody dilution from 1:200 to 1:250, the goal was to help reduce any unspecific binding and reduce background "noise". An attempt to include a negative control was performed, by omitting the primary antibody in parallel to samples where the primary antibody was included. By excluding the primary antibody (His-tag mouse monoclonal antibody), the expected results were that incubation with the secondary antibody (goat-anti-mouse IgG) would not yield any labeling with protein A-gold, as secondary antibodies should not be able to bind to *F.n.n.* on its own. Excluding the primary antibody did not yield the desired results as some labeling were still detected for the *F.n.n.* negative controls (Figure 12B, D, F, H and J). Omitting the primary antibody, should in theory be an optimal negative control, as no secondary antibody should be able to bind to *F.n.n.* and following, no gold labeling should be present. However, since some labeling is still detected, this questions the experimental setup, rather than the negative control.



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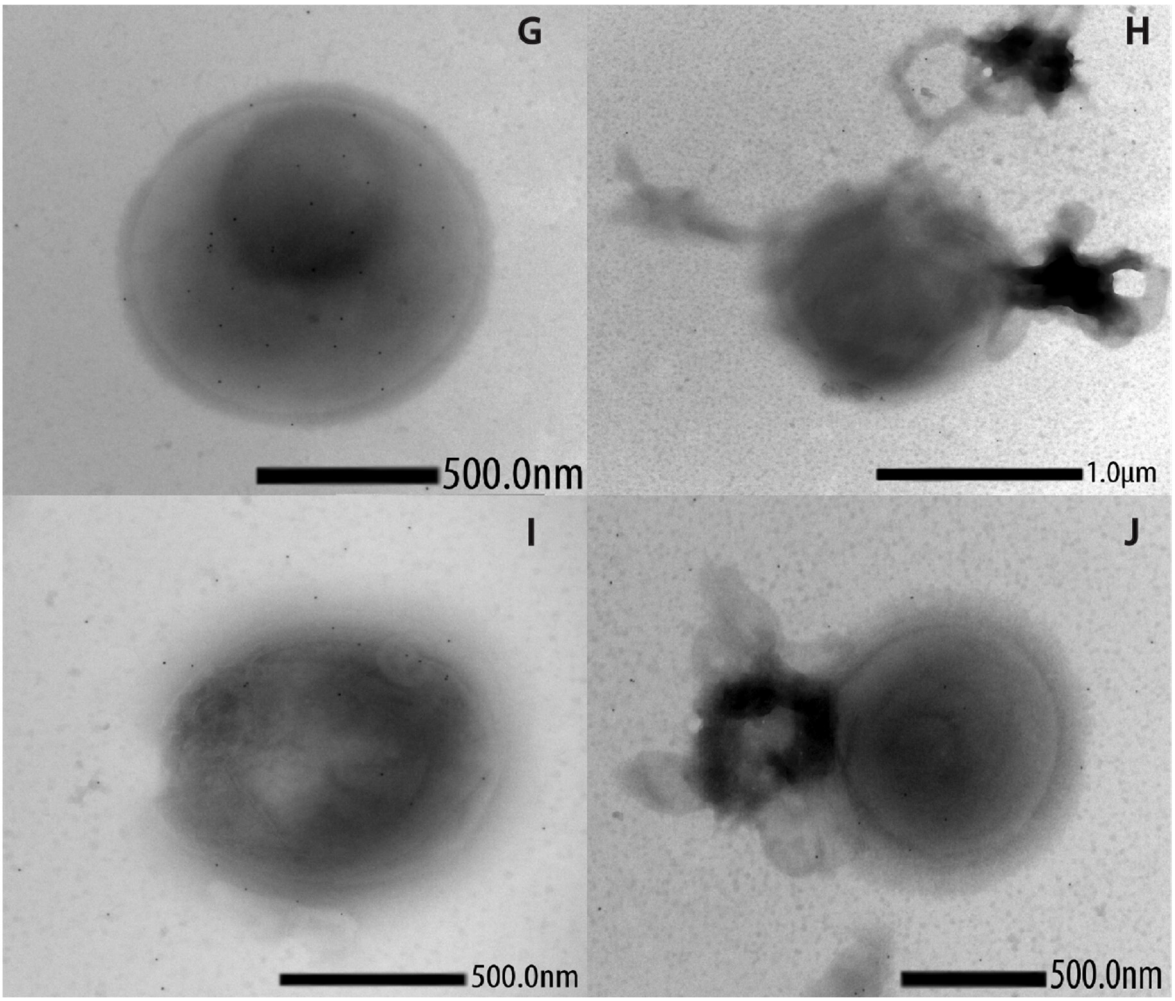

Figure 12 Immunogold labelling of F.n.n. controls and mutants. A-B: F.n.n clpB, C-D: F.n.n pilA1:his, E-F: F.n.n ΔpilD pilA1:his, G-H: F.n.n pilA6:his, I-J: F.n.n ΔpilD pilA6:his. Left column are F.n.n. treated with both primary antibody (His-tag mouse monoclonal antibody) and secondary antibody (goat-anti-mouse IgG). Right column are negative controls treated with only secondary antibody (goat-anti-mouse IgG).

As seen in Figure 12, the amount of labeling is not significantly different compared to their respective negative controls. The *F.n.n. clpB* control showed a clear degree of labeling when both primary and secondary antibody was used, as well as when the primary antibody was omitted (Figure 12A and B). The *F.n.n. clpB* should not be labeled in either case as no pili proteins are his-tagged. Very little labeling were seen for the *F.n.n. pilA1:his* mutant (Figure 12C), and could not be distinguished from the amount seen on the negative control (Figure 12D). The same was true for the *F.n.n. ΔpilD pilA1:his* mutant (Figure 12E and F). The *F.n.n. pilA6:his* mutant showed a higher amount of labeling (Figure 12G) compared to its negative control (Figure 12H). This was also true for the *F.n.n. ΔpilD pilA6:his* mutant (Figure 12I and J). Some degree of labeling on the negative controls may be explained due to unsuccessful cleaning of excess antibodies or gold particles. However, due to the low amount of labeling

seen on the positive samples, it is hard to distinguish any background "noise" from true antibody labeling.

In an attempt to solve the problem of unspecific binding of gold labelling for *F.n.n.*, controls were made by utilizing *Neisseria gonorrhoeae* (*Ng*) with and without his-tagged pili. Such experiments has been published before (162), but could unfortunately not be successfully repeated. The positive controls (*Ng* with his-tagged pili) were successfully labeled to a great extent with protein A-gold when using both primary (His-tag mouse monoclonal antibody) and secondary antibody (goat-anti-mouse IgG) (Figure 13A). However, the negative controls (*Ng* without his-tagged pili) were also heavily labeled when incubated with primary and secondary antibody (Figure 13D). As this control should not contain his-tagged pili, the primary antibody should not be able to attach to the pili of *Ng* and not yield any labeling in incubation with secondary antibody and protein A-gold. In addition, heavy labeling was seen on *Ng* without

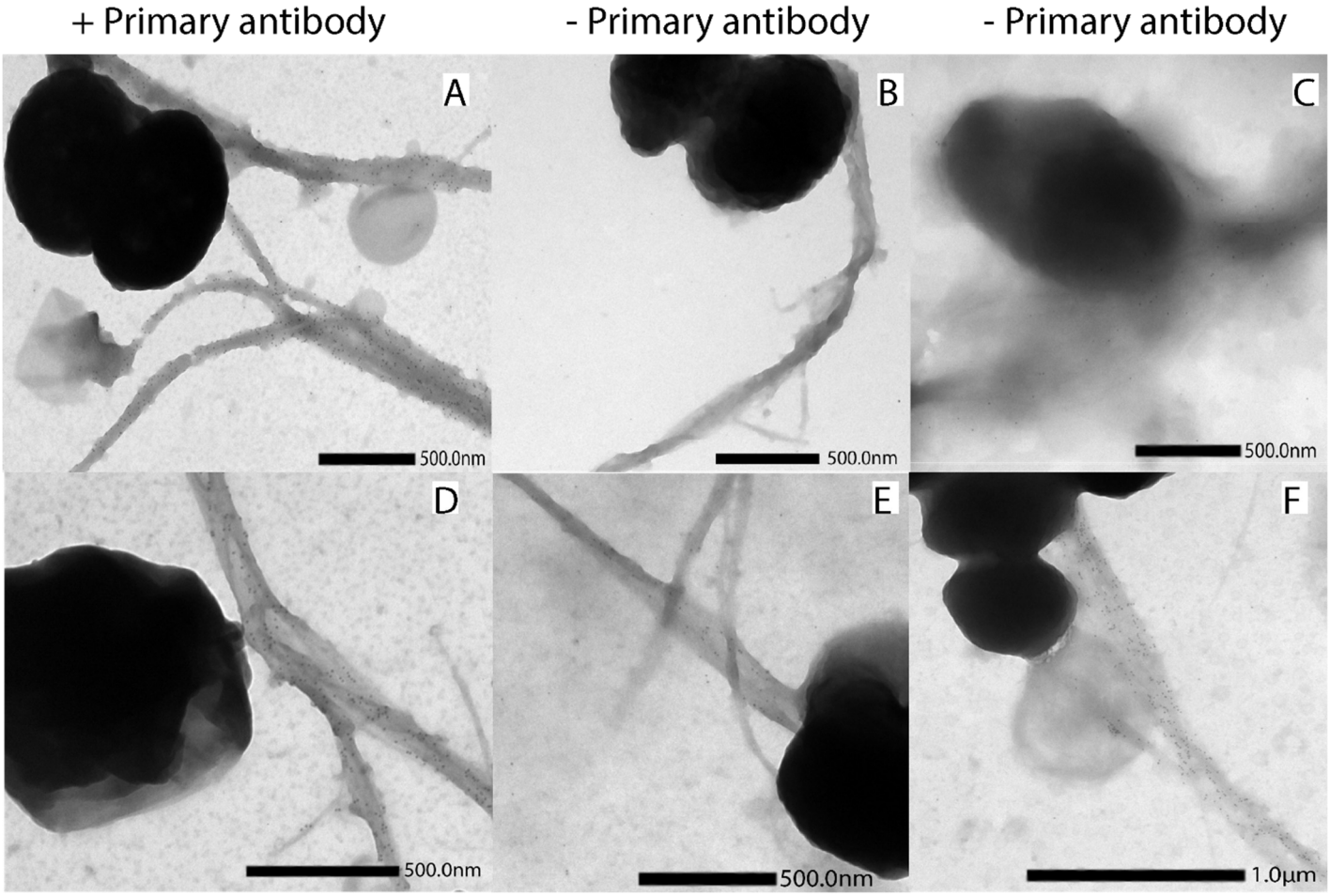

Figure 13 Immunogold experiment (improved protocol) done with Neisseria gonorrhoeae. A, B and C: Ng KS538 with 6his-tagged pili. Shows heavy labeling (A) when incubated with both primary antibody (His-tag mouse monoclonal antibody) and secondary antibody (goat-anti-mouse IgG). Little labeling was seen for the major part of the bacteria when primary antibody was omitted (B), but some labeling was on a few Ng (C). D, E and F: Ng N400 without his-tagged pili. Shows heavy labeling (D) when incubated with both primary antibody (His-tag mouse monoclonal antibody) and secondary antibody (goat-anti-mouse IgG). Heavy gold labeling was still seen when primary antibody was omitted (E and F).

his-tagged pili when primary antibody was omitted (Figure 13E and F), and to some extent on some *Ng* with his-tagged pili (Figure 13B and C). Due to these reasons, *Ng* were not used as neither positive nor negative control for further analysis.

3.2.2 Positively his-antibody labeled *Francisella* sorted by flow cytometry shows decreased labeling

The presence of pili on the surface of *F.n.n.* could be a rare event as previously published (151). Thus, an attempt was made to sort out potential positive Tfp expressing *F.n.n.* Sorting was performed by utilizing antibody against the his-tag (THE™ His Tag Antibody [iFluor 488], mAb) of his-tagged pili proteins of *F.n.n.,* attached to a fluorophore. Presence of *F.n.n.* were determined by constructing density plots, by plotting the forward scatter (FSC) on the x-axis vs. side scatter (SSC) on the y-axis. Identification of *Francisella noatunensis* subsp. *noatunensis* (*F.n.n.*) population were done by evaluating the plots made by utilizing control *F.n.n. clpB* stained with both propidium iodide (PI) and fluorescent antibody, only antibody, only PI and none of them (results not shown). A reoccurring area of density were determined to correspond to single *F.n.n*. bacteria (Figure 14A). This area was chosen and gated towards PI-positive and His-positive (Figure 14F), and PI-negative and his-positive (Figure 14G) to include all his-positives bacteria (Figure 14E), live or dead. The total amount of his-positives was determined based on amount of bacteria labeled vs. total amount of bacteria counted within the gated areas (Figure 14B). PI were used to quantify viable bacteria, as PI only binds to available dsDNA, which is only present upon lysed bacteria, as PI does not cross the membrane. This was to make sure that the percentage of his-labeling were not solely due to the presence of lysed cells, as these in some cases showed a higher degree of gold labeling after immunogold treatment.

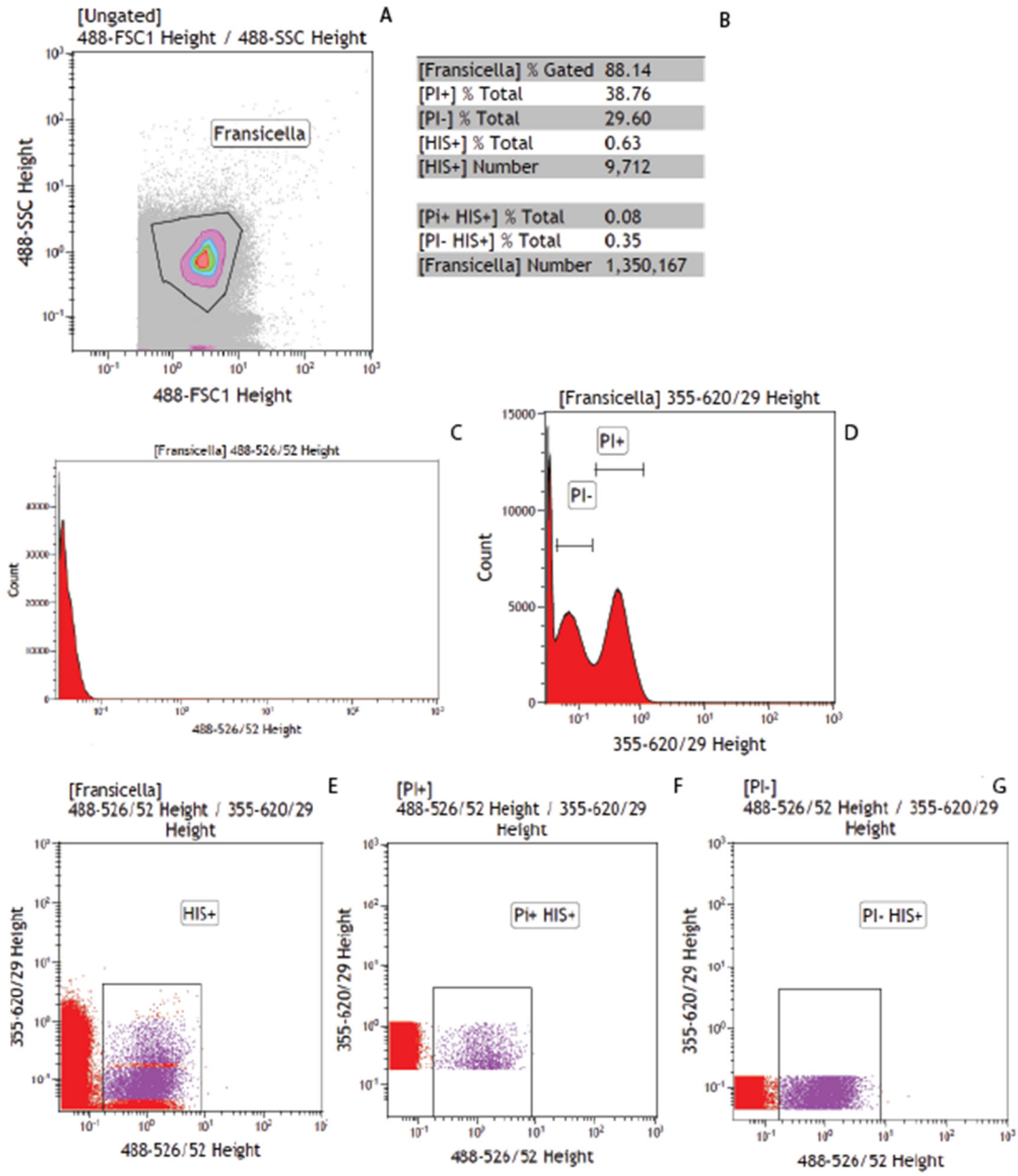

Figure 14 Flow cytometry analysis of F.n.n. strains. A: data obtained from unsorted mutant F.n.n. pilA6:his by plotting FSC (x-axis) vs SSC (y-axis). The selected area is drawn based on the reoccurring density plot (seen in color) which based on controls and actual samples are the presence of single F.n.n. bacteria. B: Shows the total amount of F.n.n. within drawn area and percentage of anti-his antibody tagged F.n.n. ([HIS+]% Total). C: Amount of anti-his antibody labeled Francisella vs. signal intensity registered with 488-526 filter, retrieved by gating the marked area in A. D: Amount of Francisella with and without PI marking. Brackets on top indicates negatively (PI-) and positively (PI+) stained bacteria. E,F,G: marked area in A is gated towards total positively anti-his antibody labeled bacteria, only PI positive and only PI negative bacteria, respectively. A larger amount positively labeled bacteria is seen for the PI negative, indicating that labeling is not solely due to lysed cells.

Assessment of percentage of labeled bacteria showed that the *F.n.n. pilE2:his, -4:his* and *-5:his*, mutants showed no labeling upon incubation with the anti-his antibody (Table 23). This was also true for the *pilE5* his-tagged mutant, which also contained a disrupted PilD type IV pili component. However, the mutants with his-tagged prepilin *PilA1* and *PilE6* and their *ΔPilD*-mutated counterparts, showed clearly, although to a small degree above zero labeling when incubated with the anti-his antibody.

Table 23 Total of his-antibody labeled bacteria in percentage, before sorting.

***Francisella noatunensis subps. Noatunensis* mutant**	**PI-pos. and His-pos. (% total)**	**PI-neg. and His-pos. (% total)**	**Total amount of His-labeled bacteria**
F.n.n. clpB	0.00	0.00	0.00
F.n.n. pilE2:his	0.00	0.00	0.00
F.n.n. pilE4:his	0.00	0.00	0.00
F.n.n. pilE5:his	0.00	0.00	0.00
F.n.n. ΔpilD pilE5:his	0.00	0.00	0.00
F.n.n. pilA1:his	0.26	2.89	3.15
F.n.n. ΔpilD pilA1:his	0.07	1.27	1.34
F.n.n. pilE6:his	0.08	0.35	0.43
F.n.n. ΔpilD pilE6:his	0.28	2.35	2.63

The initial goal by utilizing the flow cytometry was to verify the binding of the anti-his antibody to the different bacterial mutants. It is therefore not most importantly to assess degree of amount labeled bacteria, rather than the presence of labeling in itself. A decision was made to sort out the bacteria that were positive for anti-his antibody marking for further culturing and analysis. The hypothesis behind sorting the anti-his antibody labeled bacteria, were to assess two defined questions. Firstly, by concentrating the positively labeled bacteria, the theory was that the cultured bacteria would possess a higher degree of labeling, and that overall labeling would increase compared to the unsorted mutants when repeating the flow cytometry experiment with the sorted *F.n.n*. Secondly, only a few unsorted *F.n.n.* showed a considerable amount of gold labeling after immunogold treatment. The goal was to repeat the immunogold experiment using the sorted bacteria and assess if overall labeling would be more abundant.

The sorted bacteria were plated onto ECA plates with kanamycin, and successfully managed to grow, due to the fact that they had not been fixated and should still be viable after sorting. Unfortunately due to a contamination problem that arose due to unsterile circumstances under the sorting process, the *F.n.n. pilA1:his* mutant were lost from further analysis. However, the remaining mutants were confirmed by sequencing to indeed be *Francisella* and were further used for analysis (see Appendix 5).

The sorted bacteria were treated the same way as the unsorted mutants and processed through a flow cytometry to assess the percentage of anti-his antibody labeling. However, the results showed that the presence of labeling were due to random coincidence rather than separate characteristics of these *F.n.n.,* as no labeling were detected (Table 24).

Table 24 Total of his-antibody labeled bacteria in percentage, after sorting.

***Francisella noatunensis subps. Noatunensis* mutant**	**PI-pos. and His-pos. (% total)**	**PI-neg. and His-pos. (% total)**	**Total amount of His-labeled bacteria**
F.n.n. pilA1:his	X	X	X
F.n.n. ΔpilD pilA1:his	0.00	0.00	0.00
F.n.n. pilE6:his	0.00	0.00	0.00
F.n.n. ΔpilD pilE6:his	0.00	0.00	0.00

3.2.3 Immunogold labeling was not significantly increased for flow cytometry sorted *Francisella noatunensis* subsp. *noatunensis*

As well as assessing the percentage of labeling of the sorted bacteria, it was also of interest to determine any changes in gold labeling. This was done by repeating the immunogold experiment with the sorted *F.n.n.* The optimized immunogold protocol were used for this experiment (see section 2.2.7), as for the unsorted bacteria (Figure 12, section 3.2.1). Labeling with protein-A gold were scares, and presumably intact bacteria (left and middle column, Figure 15), showed little to no labeling. Interestingly there were to some extent heavy labeling among a few *F.n.n. ΔpilD pilA1:his* and *F.n.n. pilA6:his* mutants, although this was to some degree also seen among a few unsorted *F.n.n.* (Figure 16). The condition among these heavy labeled bacteria were however not optimal, as the abundance of labeling was seen within damaged or lysed cells (Figure 16A-C and 15C and F). Compared to the reduced amount of labeling in the flow cytometry, it was not surprising that overall labeling did not significantly differ from the unsorted *F.n.n.*

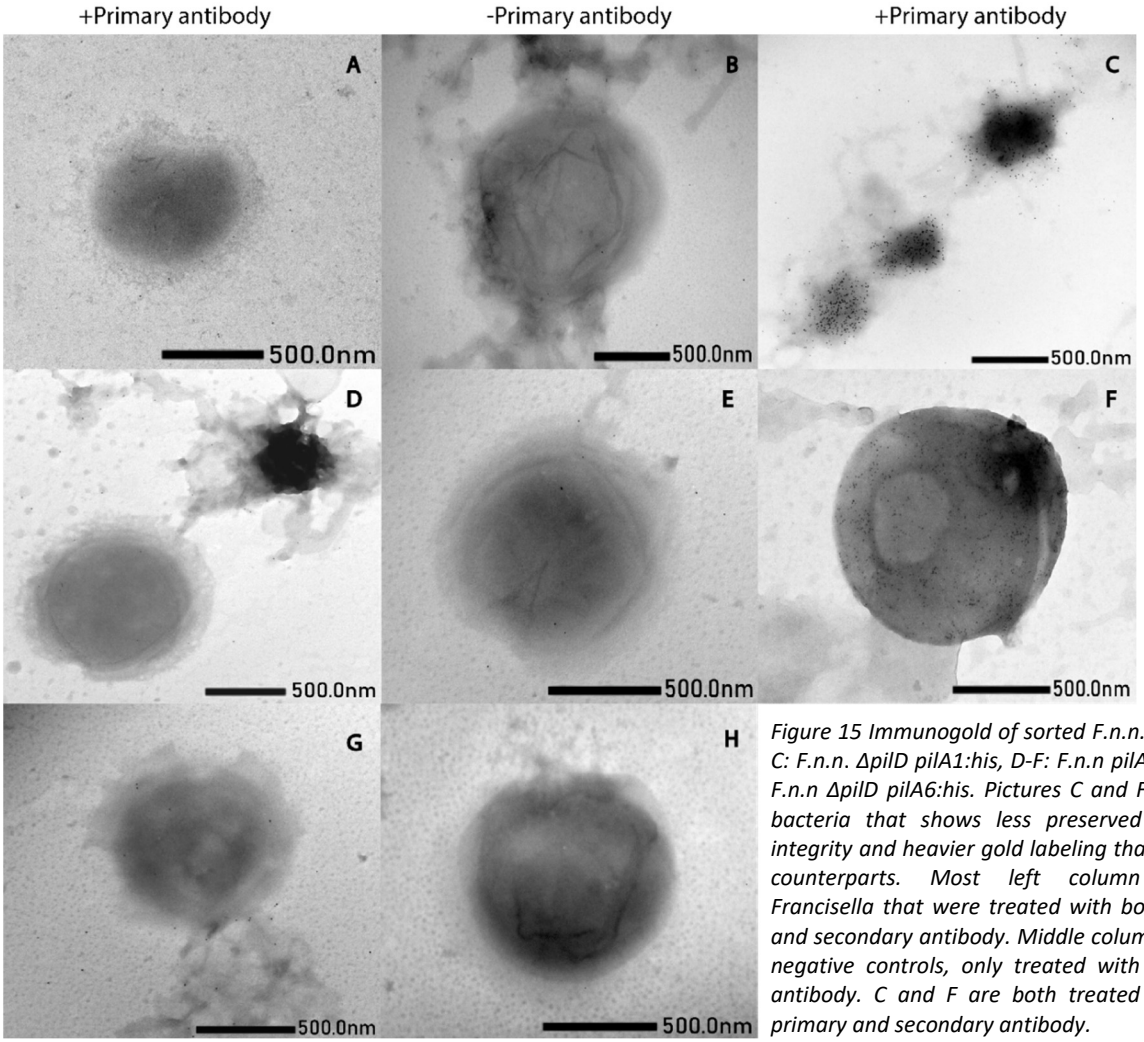

Figure 15 Immunogold of sorted F.n.n. strains. A-C: F.n.n. ΔpilD pilA1:his, D-F: F.n.n pilA6:his, G-H: F.n.n ΔpilD pilA6:his. Pictures C and F consist of bacteria that shows less preserved structural integrity and heavier gold labeling than its intact counterparts. Most left column contains Francisella that were treated with both primary and secondary antibody. Middle column contains negative controls, only treated with secondary antibody. C and F are both treated with both primary and secondary antibody.

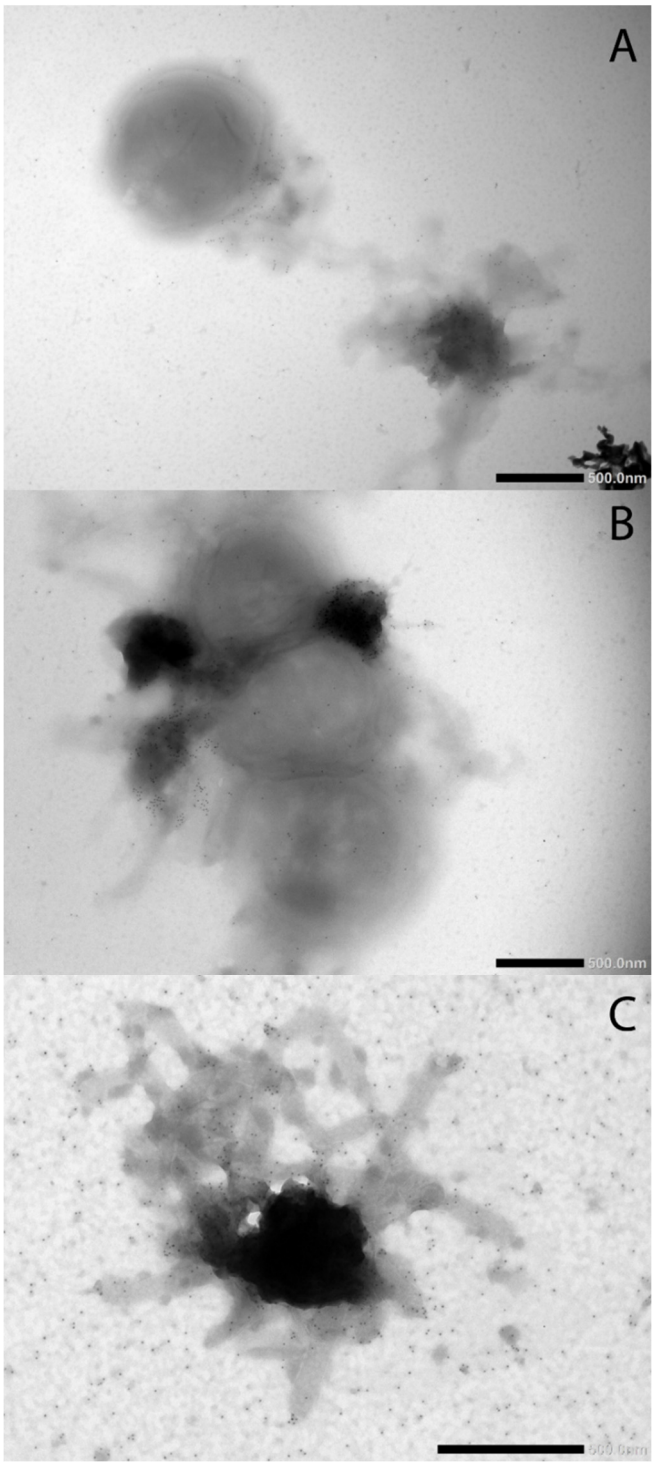

Figure 16 significant immunogold labeling among some of the unsorted F.n.n. incubated with both primary antibody (His-tag mouse monoclonal antibody) and secondary antibody (goat-anti-mouse IgG). A-B: unsorted F.n.n. ΔpilD pilA6:his. Heavy labeling is seen mostly for damaged or lysed bacteria C: unsorted F.n.n. pilA1:his. Shows heavy labeling and clear damages to bacterial structure.

3.2.4 Verifying the presence of *Francisella*

Due to possible contamination after flow cytometry cell sorting, the presence of *F.n.n.* needed to be verified before any further analysis. An attempt was made to perform colony PCR with primers specific for strain *Francisella*, but did not yield satisfactory results, as only weak bands were visible and for only some of the mutants (Figure 17). To evaluate any weaknesses in the experimental setup, *E. coli* mCherry and DH5αpir were grown and used as control. Plamids were extracted, by using the E.Z.N.A® plasmid extraction kit, from the *E. coli* and used as template in PCR with plasmid-specific primers. PCR amplification using mCherry plasmid and mCherry primers did not yield the expected size of 778bp (Figure 18, well 1). PCR amplification of DH5αpir plasmid and compl_tr_iglC primers yielded the expected product size of approx. 635 bp (Figure 18, well 4). PCR product from plasmid extracted from *E. coli* mCherry and amplified with *E. coli* m*Cherry* plasmid primers did not yield the expected size of 778bp. This is most likely due contamination or flaws in plasmid extraction. Further confirmation of this result was done by restriction cutting both extracted *E. coli* plasmids using restriction enzyme SpeI (results not shown). The correct plasmid size was only seen for *E. coli* DH5αpir, and no results were seen for mCherry, which is not surprising considering that PCR amplification of mCherry plasmid with mCherry primers, did not yield the correct size.

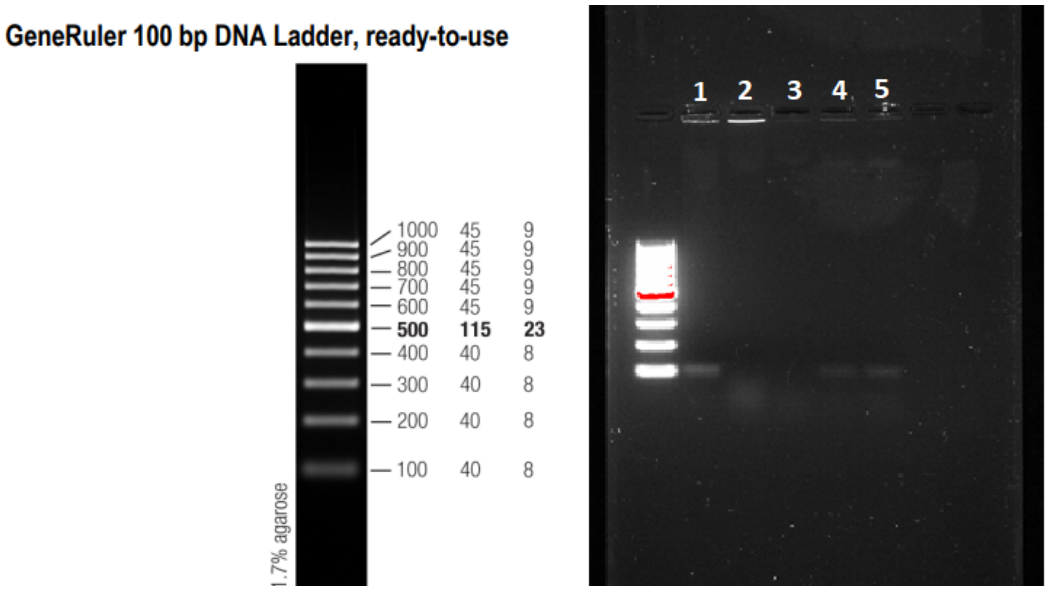

Figure 17 PCR products from colony PCR of F.n.n. using diagnostic F.n.n. primers. Wells 1-5 represents following F.n.n. mutants respectively: F.n.n pilA6:his, F.n.n. pilA1:his, F.n.n. pilA1:his, F.n.n. ΔpilD pilA1:his and F.n.n. ΔpilD pilA6:his. Bands only visible for wells 1, 4 and 5 indicating that the tested F.n.n. are of Francisella origin. Ladder: GeneRuler 100 bp DNA ladder, ready to use (Thermo Fisher Scientific). Ladder adapted from Thermo Fisher Scientific. Only one exemplary picture is shown, though several attempts were made.

An attempt was made to increase the amount of cycles in the PCR as well as DNA extracting of *Francisella* to improve the amount and quality of template, though this did not yield any advances (results not shown).

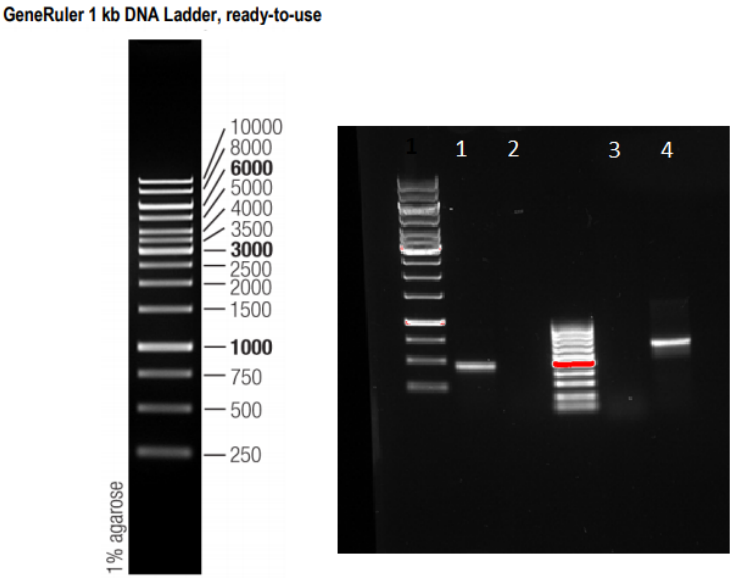

Figure 18 PCR products from plasmid extract from E. coli mutants. Wells 1-2 contains mCherry with mCherry and compl_tr_iglC primers respectively. Wells 3-4 contains DH5αpir with mCherry and compl_tr_iglC primers respectively. Ladder: GeneRuler 1kb and 100 bp DNA ladder, ready to use (Thermo Fisher Scientific). Ladder adapted from Thermo Fisher Scientific.

Because troubleshooting proved to be time-consuming and lack of improvement was seen when adjusting PCR or sample preparation protocols, the bacteria were sent to Eurofins for GATC services- LightRun tube sequencing. An excerpt of the results can be found in Appendix 5. Several samples of different content and conditions were sent for sequencing and at least one sample of each bacterial mutant were proven to be *Francisella* and was determined to be sufficient proof for use in further analysis.

3.2.5 No detectable pili in an atomic force microscope

AFM was used as a secondary method for visualizing any possible presence of outer membrane structures that could be related to a type IV pili system in *F.n.n*. As AFM is relatively time consuming, only *Francisella* mutants sorted by a flow cytometry were used. The reason for this was that by sorting out the anti-his labeled *Francisella,* the amount of pili-expressing *F.n.n.* would hopefully increase and easily detected in the AFM. As very little *F.n.n.* showed anti-his labeling, the amout of sorted bacteria were probably very limited. Very few bacteria were seen in the AFM. Figure 19A shows *F.n.n. clpB* which were prepared for flow cytometry analysis, but were not sorted. Figure 19B and C shows *F.n.n. ΔpilD pilA1:his* that were both prepared for flow cytometry analysis and sorted by utlizing the sorting mechanism of the flow cytometry. Figure 19B shows one of few structures present. Another circular object was also seen in the same sample, although the shape and size is more similar to that of an MV or artifact than to a bacteria (Figure 19C). However, no apperent pili or pili-resembeling structures were seen. Since this sample was sorted based on the presence of his-tagged pili proteins, one would excpect that the few bacteria present could contain pili-like structures, if present. No Tfp structures were observed for any of the samples. Thus, any further analysis using AFM were determined to be redundant for the purpose of this thesis. However, an interessting observation is the purity of the sample collected from the flow cytometry. Even though there were few bacteria present, the amount of debris and impurities were aslo remarkably low compared to the *F.n.n. clpB* control which had not been sorted. Yet outside the scope of this thesis, the purity of the samples collected using the sorting mechanism in the flow cytometry, is noteworthy.

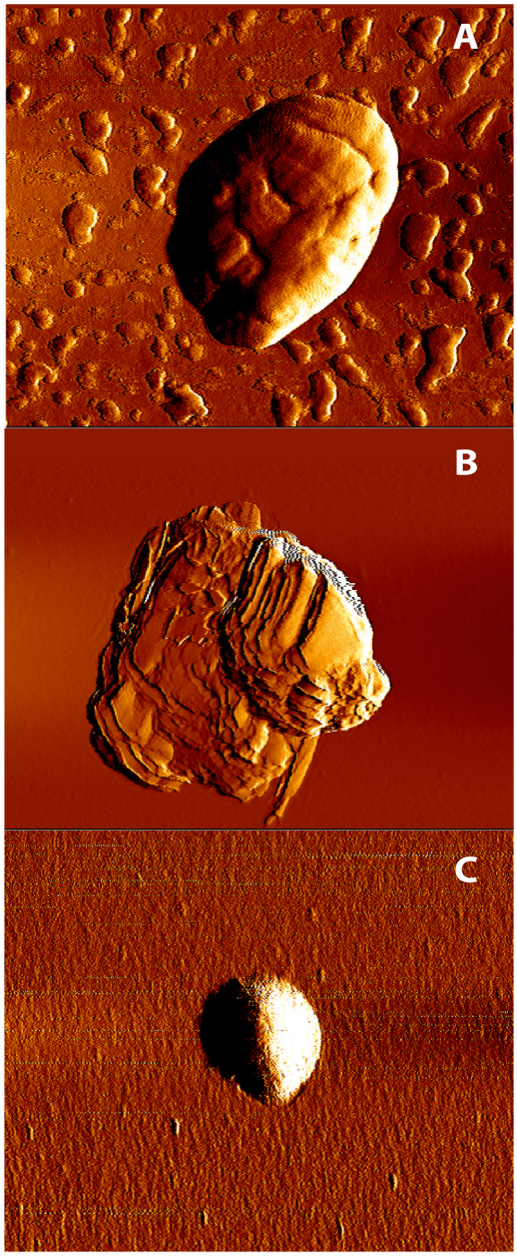

Figure 19 A: Atomic force microscopy of F.n.n. A: unsorted F.n.n. clpB. Both bacteria and possible cellular debris is seen. B and C shows F.n.n. ΔpilD pilA1:his, B is most likely a single F.n.n. while C is possibly an MV or artifact. Pictures taken with help of Ida Kristin Hegna at the Pharmaceutical department of the University of Oslo.

4 Discussion

Aquaculture is one of the fastest growing industries globally. Its importance is far from negligible and represents close to 50% of total global fish production (FAO). Even though salmon production does not account for the majority of fish farming world-wide, salmon is a high end market product and neither market value nor demand shows signs of weakening (1). To keep up with this fast growing industry, prevention of diseases common in aquaculture is of major importance. Piscirickettsiosis and francisellosis are well known diseases in aquaculture. Outbreaks are known to cause substantial economic losses and are linked to consequential increase in use of antibiotics. The surge in use of antibiotics is mainly due to lack of long-time protective vaccines or an absence of available vaccines in general, as for francisellosis. A common vaccine strategy is to use non-replicating subunits from the pathogen. This method depends on having sufficient knowledge of the bacterial pathogenicity and virulence factors. In this thesis, *P. salmonis* and *F.n.n.* were used to evaluate and characterize (respectively), virulence factors, which may be applicable in vaccine development.

4.1 Upregulation of immune related genes in Atlantic salmon reveals possible protective effect from MV immunized groups during *P. salmonis* infection

Several immune related genes in Atlantic salmon were examined in this thesis, with the purpose of evaluating the immunological response of a vaccine trial conducted by Dr. Alexander Kashulin in 2016. Kidney, spleen, and liver from fish vaccinated with MVs isolated from *P. salmonis* LF-89 and infected with *P. salmonis* EM-90 were harvested, processed and analyzed. The gene expression in the untreated group and vaccine trial groups were compared to the control group which was injected with saline (placebo).

From the results (section 3.1.1) a change in gene expression were seen in spleen and kidney at 0 WPC, and in all tissues investigated at 4 WPC. Five of the seven genes investigated is reoccurring upregulated in the different tissues and in the immunized groups. At the two different time points and in the three different tissues, a statistically significant fold increase in gene expression was detected in the 100 µg MV group, untreated group, and 40 µg MV + adjuvant group. No changes in gene expression were seen in the adjuvant group. Adjuvants are

usually added to vaccines to act as supplementary immune stimulating compounds, as well as functioning as a delivery mechanism of the vaccine component itself. However, adjuvants are on their own effective immune stimulators. Nevertheless, no statistically significant changes were detected for any genes in this group, when compared to control (saline injected). What one can see based on this group's immunological response is that the adjuvant itself is most likely not contributing on its own to the response detected in the 40 µg MV + adjuvant group. However, these results needs to be viewed in the light of the combination of adjuvant and antigen given, in which the response may not be exclusively contributed to neither the concentration of MV administered nor the addition of adjuvant, but rather the possible effect they give in combination.

An increase in gene expression SOCS was detected in kidney of the 100 µg MV group at 0 WPC. This key regulator of growth factor and cytokine signaling is believed to play an inhibitory role in JAK-STAT signaling, a signal transduction pathway important in controlling transcription (53). JAK-STAT plays a role in cytokine receptor signaling, and enhanced inhibition by increased SOCS expression, hence reduces cytokine-signaling ability. As cytokines plays an essential role in the initiation of an immune response, the increased expression of SOCS may indicate a physiological state, where less transcription of their downstream effectors is needed. As this is true only before infection with *P. salmonis*, this may be an effect caused by the immunization itself.

Gene expression of IL-8 was increased in kidney and spleen at 0 and 4 WPC, respectively. At 0 WPC, this was detected in the 40 µg MV + adjuvant group, while in the 100 µg MV group at 4 WPC. IL-8 is an important chemokine, and is proven to both recruit and guide neutrophils to infection sites (70). An increase in gene expression of IL-8 might therefore be linked to an activation of the innate immune response. As fish at 0 WPC are not infected with *P. salmonis*, this fold increase might be linked to the immunization (performed 7 weeks before). However, the increase in expression seen at 0 WPC in the 40 µg MV + adjuvant group is neither maintained nor statistically significant at 4 WPC. Interestingly, at 4 WPC IL-8 expression is statistically significant compared to the control group, in spleen of the 100 µg MV group. However, as in the kidney, expression of IL-8 was higher at 0 WPC, but not statistically significant, indicating that expression of IL-8 decreased during infection with *P. salmonis.* This trend of decrease in IL-8 expression after infection was also seen within the liver, although not statistically significant. As IL-8 is part of the innate immune response, which often acts in first

response to intruding bacteria or PAMPs, the time points in which samples were harvested for immunological analysis needs to be taken into account. Since the expression of IL-8 seems to decrease after challenge with *P. salmonis*, one could speculate if this is related to the possible immune modulatory effects induced by *P. salmonis* itself. In reality, as expression were only checked at one time point after challenge (4 WPC), it is not unlikely that data has been lost in the early infectious stage, which could have given a more thorough insight into immune expression in the early disease stages. It is therefore not entirely inconceivable that an initial upregulation in expression of IL-8 may have taken place before the observed reduction, but this cannot be determined on the reduction in expression alone.

An increased IL-12 expression was seen in the 40 µg MV + adjuvant group in spleen and liver at 0 and 4 WPC, respectively. IL-12 is an important pro-inflammatory cytokine for Th1 cells, and is known in humans to initiate differentiation of naïve T cells into memory T cells and cytokine-producing Th cells (72). The function of IL-12 in teleost is also believed to be related to Th cell development (74) . As *P. salmonis* are intracellular bacteria, the activation of a range of immune responses is needed to successfully eradicate an infection, as antibodies alone is not sufficient (168). However, though the expression of IL-12 is increased in spleen for the 40 µg MV + adjuvant group at 0 WPC, this is not seen 4 WPC. The opposite is true in the liver in which IL-12 expression is increased at 4 WPC in the 40 µg MV + adjuvant group. This is also the case at 4 WPC in the kidney of fish from the 100 µg MV group, in which expression of IL-12 is statistically significantly increased at 4 WPC. In a study on *in vitro* infection of *P. salmonis* in a salmonid cell line conducted by Álvares et al., (169) it was discovered that *P. salmonis* manipulated host cytokines IL-12 and IL-10. An upregulation of the pro-inflammatory IL-10, and a decrease in IL-12 expression was seen, and is suggested to be a mechanism which promotes intracellular replication of the pathogen (169). No statistically significant decrease/increase was detected for the IL-10, however an increase of IL-12 expression was seen in both 40 µg MV + adjuvant, and 100 µg MV group, which might indicate a protective effect, as infection did not reduce IL-12 expression.

Another result which further strengthens the hypothetical possibility that the immunized groups might have induced a protective effect in Atlantic salmon, is the expression of MPEG1. MPEG1 encodes a MACPG, perforin-2, which has been proven to play an important role in intracellular destruction of pathogens (60). A statistically significant increase in MPEG1 expression was seen in spleen of fish from the 40 µg MV+ adjuvant group at 4 WPC. This enhanced ability of

intracellular destruction of *P. salmonis* might possible be a protective effect elicited by the vaccine given. As the same effect was not seen in the 100 µg MV in spleen at 4 WPC, one may speculate if this protective effect is due to the combination of the vaccine and adjuvant, rather than MVs alone.

MHCIIB are molecules important in initiating immune responses. These complexes are responsible for extracellular antigen representation during an infection, which binds and stimulates CD4+ helper T cells and downstream Ig and IFN-γ activation of macrophages (63, 64). A statistically significant increase in MHCIIB expression was seen within the 100 µg MV group at both 0 and 4 WPC. In kidney, MHCIIB expression was significantly increased at both 0 and 4 WPC (2.07 and 1.76 fold increase in 0 and 4 WPC, respectively). This observation agrees with the possible protective properties seen for other genes in the MV based vaccine groups. There is an agreement in several papers investigating the vaccine requirements necessary to combat intracellular bacterial infections, that antibodies might not be sufficient on their own to fight these pathogens, in which T cell activation may be of importance (13, 38, 93, 168).

Despite the possible small protective effect observed in the immunized groups, another issue was the difference in expression of genes, comparing the untreated group to the control group. As the only difference between the two groups are injection of saline in the control group, one would have expected that no substantial difference in gene expression should be present. However, this is not the case. A statistically significant fold increase in gene expression was seen within SOCS, IL-8, MPEG1 and MHCIIB. No connection was seen between any of these genes, as none of the same genes was simultaneously significantly changed neither at the same time point nor between tissues. SOCS was the only gene in which a significant increase in gene expression was seen two times, but in different tissues and at different time points (kidney at 0 WPC, and spleen at 4 WPC). Because the difference between these groups are the injection of saline in the control group, this is probably also the reason for these discrepancies. This may be due to a low number of biological replicates, giving insufficient power to the statistical analysis. The previous results should be viewed in the light of this observation, and any conclusions should be drawn carefully.

Another important factor that might be worth mentioning is the fact that different strains of *P. salmonis* were used during the vaccine trial. While the MVs was harvested from *P. salmonis* strain LF-89, *P. salmonis* strain EM-90 was used during the cohabitant infection model. Though the two strains share a 16S gene similarity of 98.5% (89), a difference was seen when comparing cumulative mortality during a cohabitant challenge in Atlantic salmon. Infection of EM-90 was shown to give a higher cumulative mortality as well as onset of death appearing earlier than for strain LF-89 (99). This finding might explain the cumulative mortality obtained from the vaccine trial (Figure 5 section 3.1) examined in this thesis, and how it might be linked to differences in *P. salmonis* strain virulence. The mortality rate obtained from the vaccine trial showed that no protective effect was obtained from immunization with neither 100 µg MV nor 40 µg MV + adjuvant. No difference in total mortality was observed between the vaccine groups, and no notably distinguishable difference were seen in onset of death. However, analysis of both IL-12 and MHCIIB gene expression shows that immunization with *P. salmonis* MVs may be able to induce a protective effect, though no protection was seen in relation to mortality.

4.2 Histological examination of kidney and spleen tissue from MV immunized fish vs. control group

To evaluate the level of disease markers present in *P. salmonis* infected Atlantic salmon, a histological examination was performed on kidney and spleen for immunized trial groups 100 µg MV, 40 µg MV + adjuvant, and control. No distinct granulomas were seen. However, several signs of disease and tissue disruptions were seen within the different groups. No data is available on whether there were any external or behavioral signs of disease on fish harvested for analysis. However, tissues from infected and non-infected Atlantic salmon could clearly be distinguished, as the infected kidney and spleen were enlarged compared to the "healthy" tissues (results not shown).

As no granulomas were seen in the infected fish, one may speculate if this is due to the strain of *P. salmonis* (EM-90) used in the co-habitant infection model. Fish infected with EM-90 has been shown to have a greater cumulative mortality and earlier onset of death, compared to LF-89 (99). For this reason, sudden death may have occurred prior to any substantial granuloma formation, and that lack of these disease markers is because of the high pathogenicity of EM-90. However, several signs of a more systemic infection were seen in infected fish for both

spleen and kidney compared to “healthy” fish. A clear distinction could be seen when comparing tissues between the infected and non-infected fish (Figure 8 and 9). One can see that tissue distribution in infected fish is less organized and is more chaotic compared to its “healthy” counterparts. In addition, the presence of enlarged nuclei is clearly seen in the spleen of a 100 µg MV infected group fish (Figure 8E). An enlarged nuclei is often seen during disease, as gene expression may be increased (170).

In addition to the chaotic tissue organization, vacuolar degeneration was also seen (Figure 10E and 8A). Vacuolar degeneration is seen as circular structures, which appear empty inside. These formations are often seen in cells after exposure to a bacterial or viral pathogen, and are often linked to cell death (171). As these structures are seen in the infected fish and not the non-infected ones, it is likely that these structures are present as a response to *P. salmonis* infection. This theory is further strengthened when looking at Figure 8A in which both vacuoles as well as melanomacrophages are seen scattered throughout the tissue, indicating disease progression. In Figure 10F, a larger patch of disrupted tissue organization is seen. Though no vacuoles are seen, this is most likely a tissue inflammation. As necrosis of hematopoietic cells is often followed by formation of granulomas, the tissue degenerations seen in Figure 10E and F may all be precursors of granulomas. However, as these phenomenon is only seen in the infected fish, one may conclude that these structures are present in the tissue due to *P. salmonis* infection.

The difference in amount of melanomacrophages present, is clearly distinguishable between non-infected and infected fish in the spleen (Figure 8). However, the difference in amount of melanomarophages present was less prominent in the kidney. No clear distinction could be made between infected and non-infected samples, as presence of melanomacrophages were abundant in all samples (Figure 9). However, the tissue inflammation seen in Figure 10F shows a local accumulation of melanomacrophages. Even though the presence of melanomacrophages cannot be exclusively linked to infections based on the amount present, their close proximity to the inflammation site and how they surround it is worth noting. Melanomacrophages have been proposed to possess important functions, both immunological as well as non-immunological. Increase in size as well as frequency of these structures is associated with degeneration of haemopoietic tissue, which is common during *P. salmonis* infection (46). Based on these observations, it is likely that the increased presence of melanomacrophages may be linked to infection, especially in the spleen, as these are more abundant in infected fish.

4.2.1 Histological examination showed a possible presence of residual adjuvant in blood vessel of spleen

As seen in Figure 10A, B, and C, a structure is present within one of the blood vessels in the spleen of the 40 µg MV + adjuvant group (at 4 WPC). During a meeting with Prof Erling O. Koppang (NMBU), it was suggested that this structure might be residual adjuvant. The adjuvant used in this vaccine trial were Montanide ISA 780 VG, which is a water-in-oil (vegetable oil) formulation used to achieve long-term protective immune response (Seppic). Adjuvants are useful supplementary compounds in vaccines, but are also linked to adverse effects, however this is most often seen among mineral oil adjuvants (172). Even if this substance is residual adjuvant, no other evidence is present that can directly link any tissue degeneration or inflammation to this occurrence. However, for the purpose of this thesis it is worth pointing out that granulomas can be hard to distinguish from other structures (for an untrained eye). Advanced granulomas may contain a central area of mineralization as well as being well confined, which in some cases may be confused with vessels.

4.3 Immunogold show no definite presence of pili on *Francisella noatunensis* subsp. *noatunensis*

The presence of a type IV pili has earlier been characterized in *F.t.t.* Work performed in this thesis was based upon whether the Tfp system also is present in *F.n.n.*, as *Francisella* share several genetic similarities, as the e.g. FPI (130, 131). As Tfp are extracellular complexes, the study was conducted by creating mutants in which possible extracellularly located subunits of the system were his-tagged. An anti-his antibody was used to perform both immunogold as well as flow cytometry to evaluate the presence of this structure.

Before utilizing a flow cytometry, the presence of pili was examined by performing immunogold labeling and visualized by employing TEM. The results obtained from this experiment were inconclusive, as labeling were to some extent seen also in the negative controls. However, it can be concluded that no evident characteristic pili were present in any of the *F.n.n.* strains examined. An attempt was made to include *Neisseria gonorrhoeae*, with his-tagged pili as a positive control for the immunogold experiment. Due to unknown reasons, there was a substantial amount of gold labeling on *N. gonorrhoeae* without pili his-tagging. Several attempts were made, though all came up empty handed. Due to time constraints, it was decided that his-tagged *N. gonorrhoeae* was not going to be used as positive control. However, negative

controls had been conducted in parallel in the experimental set-up, in which incubation with primary antibody was omitted. Some gold labeling were still present in some *F.n.n. clpB* controls as well as in the mutant *F.n.n.* controls without primary antibody. However, it was decided that a substantial amount of effort had been put into creating an optimal negative control, and that the results obtained were rather to be discussed in detail.

Due to the presence of some gold labeling on the negative controls used in the experiment, it is hard to distinguish any clear separation between positively labeled mutant *F.n.n.* and background. However, despite this low-level marking seen on most of the mutants observed, gold labeling were abundant in a few samples. This was true for both unsorted *F.n.n.* mutants *F.n.n.* Δ*pilD pilA6:his* and *F.n.n. pilA1:his* (Figure 16) as well as for some sorted mutants *F.n.n.* Δ*pilD pilA1:his* and *F.n.n. pilA6:his* (Figure 15C and F). Interestingly, the common trait between these extensively marked bacteria is their condition, in which all heavily marked bacteria show signs of, or is clearly lysed. Because of this, it was decided that mutant and control *F.n.n.* were to be analyzed in a flow cytometry to be able to assess the possibility that heavy labeling is solely due to exposing of the intracellular content.

4.4 Sorting of his-tagged *Francisella* lowered the level of anti-his antibody labeling

A flow cytometry was used to assess both the amount of mutant *F.n.n.* labeled with the anti-his antibody, as well as determining if gold labeling was due to exposure of the intracellular content. Evaluation of the physical state of the bacteria, were performed by using PI staining, which only stains lysed bacteria. PI only binds to dsDNA, which is only available when bacteria is lysed, as PI does not cross the bacterial membrane. In this way, anti-his antibody labeled bacteria could be separated in the flow cytometry based on fluorescence characteristics of PI and the antibody. However, as seen in Table 23 (section 3.2.2), the distribution of anti-his antibody labeled bacteria were not exclusively seen for the PI-positive *F.n.n.* From these results alone, it is not conclusive whether labeling is solely due to the presence of lysed bacteria.

As anti-his antibody labeling was seen for only some of the *F.n.n.* mutants, it was of interest to examine these on their own. This was possible by sorting out the anti-his antibody labeled bacteria which were also PI negative and grow these one separate ECA plates for further analysis. There were two main goals for the sorting of the positively anti-his antibody labeled bacteria. The first goal was to assess any physical differences between the sorted bacteria and

unsorted. This was done by repeating the immunogold labeling protocol for the sorted bacteria and comparing the amount of labeling and physical characteristics to the unsorted ones. The second goal was to determine if sorting the positively labeled bacteria would increase the amount of labeling when repeating the flow cytometry experiment with the sorted bacteria.

As for the unsorted bacteria, no significant gold labeling was seen for the sorted bacteria compared to the controls (Figure 15). However, as for some of the unsorted bacteria, a significant increase of labeling was seen for bacteria which appeared lysed when viewed in a TEM (Figure 15C and F). When repeating the flow cytometry analysis with the sorted bacteria, no anti-his antibody labeling was detected. This result was puzzling, as one would expect at least a low-level of labeling present, as seen for the unsorted bacteria, if this is a trait that is only needed by a fraction of the population. As pili are not commonly seen on *Francisella*, but have been confirmed on some strains, it is possible that the lack of pili could be due to unsatisfactory environmental conditions, in which pili expression may not be present. In addition, histidine-tagging of pili proteins has been shown to both affect cell growth and the ability to assembly pili (162). A complete loss of labeling overall indicates that the sorted bacteria may after all not have been positively labeled. Another probability is that something might have happened during the analyzing process, in which a possible external pili subunit expression was lost. This may be due to both random mutation as well as possible genetic disturbance or impact by the histidine-tag on the pili protein.

It is not uncommon that bacteria that is prone to serial passage and exposed to environmental stress may lead to loss of or mutations in genes (173). It is therefore possible, but unlikely that the labeled bacteria originally may have expressed his-tagged pili subunits on its surface, which was lost during handling, as these may not have been important for *F.n.n.* growth and survival. This hypothesis could agree with the amount of labeling seen, in which <4% labeling may indicate a non-essential function among the bacteria, or suboptimal environmental conditions for pili expression. The chemicals used to label, and the following sorting, may also have been too overwhelming and caused all surviving bacteria to have lost the ability to express the his-tagged pili subunit. However, the sorted bacteria were directly prepared and viewed in an atomic force microscope after sorting, in which no pili structures were visible. Due to the amount of labeling seen in the flow cytometry before sorting, the next step in analysis would be to evaluate the genetic presence of both his-tag and pili proteins in the sorted *F.n.n.* The reduced labeling seen after sorting could be due to loss of the histidine-tag, as well as possible

loss of the ability to assembly pili, if *F.n.n.* in theory were able to produce Tfp. This could be done by performing a western blot with specific 6-his antibodies.

Gold labeling were seen at some of the controls as well as mutants, and to an abundant amount on some lysed bacteria. Bacterial colonies and cultures of *F.n.n.* are quite "sticky", which is consistent with the fact that creation of biofilm and possession of an outer capsule is a normal trait seen among several *Francisella* (174). This stickiness caused several problems when using the *F.n.n.* for flow cytometry analysis (the bacteria tended to stick together and get stuck in the tubing of the instrument). A possibility may therefore also be that the gold particles seen during immunogold labeling, is due to the stickiness of the bacteria itself. This may be further enhanced when cytosolic content is leaked in which antibodies may get tangled and give rise to fake-positive gold labeling or the gold itself gets stuck. However, the low-level labeling seen for the control may also be due to unsuccessful washing, and some background is therefore to be expected.

5 Conclusion

This study investigated the immunological response of a vaccine based on membrane vesicles from *Piscirickettsia salmonis* against piscirickettsiosis, as well as assessing the possible presence of a type IV pili system in *F.n.n.*

The MV based vaccine examined showed some possible protective abilities. This observation is based on the increase in gene expression detected for IL-12, MHCIIB and MPEG1 in the immunized groups. However, the discrepancies between the untreated and control group needs to be taken into account and any clear conclusion cannot be drawn. The results from this work may help in further improving immunization against *P. salmonis* utilizing MVs.

The degenerative characteristics and increased presence of melanomacrophages can be attributed to the *P. salmonis* infection. This observation is supported by the comparison between non-infected and *P. salmonis* infected fish, in which the only infected fish were affected.

No pili were visible when examining *F.n.n.* in neither EM nor AFM. However, some gold labeling were present on the surface of mutant *F.n.n.*, but the amount could not clearly be distinguished from the control. Sorting of the positively anti-his antibody labled *F.n.n.* using a flow cytometry did not increase the amount of labeled bacteria. Only damaged or lysed bacteria showed a consistent immunogold labeling, indicating either an intracellular presence or accumulation of antibodies or gold in these “sticky” areas. However, analysis from the flow cytometry showed that labeling of his-tagged structures were present on both intact as well as lysed cells. This observation is supported by the use of propidium iodide, which can only penetrate damaged or lysed cells.

6 Outlook

The original plan was to evaluate the presence of *P. salmonis* in infected Atlantic salmon tissue by performing immunohistochemistry, but this fell through due to SARS-CoV-2. This additional analysis would allow an extensive examination of the amount of *P. salmonis* present, and if there is a link between the amount of pathogens present and the mortality rate. A plan was also made to assess the presence of antibodies in serum collected from Atlantic salmon, but this fell through due to time constraint. This would be a good experiment to evaluate the efficiency of the MV based vaccines, and possibly support the findings that the vaccine itself may have provoked an immunological response. An attempt was also made to include primers for interferon-γ and tumor necrosis factor α in the qPCR experiment, but due to unsatisfactory primer efficiencies and melting curves, these were omitted. These are however valuable immunological genes, that would maybe give more insight into the vaccine response.

As examined in this thesis, there is no apparent presence of characteristic type IV pili in the outer membrane of *F.n.n.* neither in EM nor in AFM. Even though some labeling were seen using a flow cytomery before sorting the bacteria, no labeling was present after. It would therefore be interesting to examine the sorted *F.n.n.* and evaluate whether the expression of the mutant his-tags are lost or if the labeling itself is due to unspecific binding. The evaluation of His-tag presence on pili proteins could be assessed by performing a western blot, using antibodies specific for the 6-His tag. It would also be valuable to include a positive control and adjust the immunogold protocol to reduce any unspecific binding present at the negative controls.

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Appendix 1: RNeasy mini kit (QIAGEN) protocol

All reaction steps were kept on ice unless specified and centrifuged at 10.000 rpm.

1. 5-10 mg of tissue were weighed out and placed in a 1.5 mL Eppendorf tube.

2. 600 µL RLT buffer were added to tube and homogenized by dispersing the tissue using a pellet pestle. Further homogenization was performed by passing the lysate through a 1 mL syringe with a 23 gauge needle to ensure proper tissue disruption.

3. Eppendorf tubes were centrifuged for 3 minutes and supernatant transferred to a new Eppendorf tube.

4. 600 µL of 70% ethanol (diluted in RNase free H_2O) were added to the supernatant, and 700 µL from this transferred to a spin column provided by the kit and centrifuged for 15 seconds.

5. Flow through were removed and previous step repeated.

6. DNA removal procedure:

    6.1. 350 µL of the RW1 buffer were added to the spin column and centrifuged for 15 seconds.

    6.2. 10 µL of Dnase I and 70 µL RDD buffer were mixed and carefully added directly to spin column membrane and incubated at room temperature for 30 minutes.

    6.3. 350 µL of the RW1 buffer were added to spin column and centrifuged for 15 seconds. Flow through were removed.

    6.4. 500 µL of the RPE buffer were added to spin column and centrifuged for 15 seconds. Flow through were removed.

    6.5. Spin column were placed into a new collection tube and centrifuged for 2 minutes, to ensure proper removal of residual liquid.

7. Spin column were placed into a 1.5 mL Eppendorf tube and 22 µL of Rnase free H_2O added carefully to membrane of the spin column and centrifuged for 2 minutes.

8. Concentration of RNA eluted from spin column in step 7 were measured in a Mettler Toledo UV spectrophotometry (LabX® Ready).

Appendix 2: DNA Mini Kit QIAMP protocol

1. Bacterial colonies from selective plates were dispersed in 1 mL of PBS in a 1.5 mL Eppendorf tube.
2. 15 µL proteinase K were added to an empty 1.5 mL Eppendorf tube.
3. 200 µL of bacterial sample were added to tube with proteinase K at room temperature.
4. 200 µL of buffer AL were added to solution and sample pulse-vortexed for 15 seconds before incubation at 56°C in a digital dry bath (Labnet) for 10 minutes.
5. Sample were centrifuged briefly.
6. 200 µL 96% ethanol were added to solution and sample pulse-vortexed for 15 seconds.
7. Sample were centrifuged briefly.
8. Sample were transferred to spin column and centrifuged at 8000 rpm for 1 minute.
9. Spin column were transferred to a new, clean collection tube.
10. 500 µL of buffer AW1 were added to spin column and centrifuged at 8000 rpm for 1 minute.
11. Spin column were transferred to a new, clean collection tube.
12. 500 µL of buffer AW2 were added to spin column and centrifuged at 14.000 rpm for 3 minutes.
13. Spin column were transferred to a new, clean collection tube before centrifuging at 14.000 rpm for 1 minute.
14. Spin column were placed into a clean 1.5 mL Eppendorf tube and 100 µL of buffer AE added directly to the column membrane and incubated at room temperature for 1 minute. Sample were centrifuged at 8000 rpm for 1 minute to elute the DNA.
15. Concentration and purity were measured in a nanodrop.

Appendix 3: E.Z.N.A.® Plasmid DNA Mini Kit I Spin Protocol

1. Isolate a single colony from a selective plate and disperse in 1-5 mL LB medium.
2. Centrifuge at 7000 rpm for 1 minute at room temperature.
3. Aspirate and discard the culture media.
4. Add 250 µL Solution I/RNase A.Vortex or pipet up and down to mic thoroughly.
5. Transfer suspension into a new 1.5 mL microsentrifuge tube.
6. Add 250 µL Solution II. Invert and gently rotate the tube several times to obtain a clear lysate and incubate 2-3 minutes if necessary.
7. Add 350 µL Solution III. Immediately invert several times until a flocculent white precipitate forms.
8. Centrifuge at 9100 rpm for 10 minutes. A compact white pellet will form.
9. Insert a HiBind® DNA mini column into a 2 mL collection tube
10. Transfer the cleared supernatant from step 8 by carefully aspirating it into the HiBind® DNA mini column.
11. Centrifuge at maximum speed for 1 minute.
12. Discard the filtrate and reuse the collection tube.
13. Add 500 µL HBC buffer.
14. Centrifuge at maximum speed for 1 minute.
15. Discard the filtrate and reuse collection tube.
16. Add 700 µL DNA wash buffer
17. Centrifuge at maximum speed for 1 minute.
18. Discard the filtrate and reuse the collection tube.
19. Centrifuge the empty HiBind® DNA mini column for 2 minutes at maximum speed to dry the column matrix.
20. Transfer the HiBind® DNA mini column to a clean 1.5 mL microsentrifuge tube.
21. Add 30-100 µL elution buffer or sterile deionized water directly to the center of the column membrane.
22. Let sit at room temperature for 1 minute.
23. Centrifuge at maximum speed for 1 minute.
24. Measure concentration and purity in a nanodrop.

Appendix 4: Primer efficiency

Table 25 Primer efficiency of primers used in qPCR

Primer set	X-value	Primer Efficiency
18S	-2,994	96.42 %
EF1α	-3,203	105.21 %
IL1B	-3,745	84.94 %
MHCIIB	-3,192	105.72 %
MPEG1	-3,150	107.71%
SOCS	-3,686	98.09%
IL-8	-3,332	99.58%
IL-10	-3,360	98.44%
IL-12	-3,697	110.17 %
IFNG*	-0,946	1040.47%
TNFα*	0,00	No results
TNFα_1*	-3,407	96.57%

*Primers that were tested but not used for further analysis due to unsatisfactory primer efficiency or melting curves.

Appendix 5: *Francisella noatunensis* subsp. *noatunensis* sequencing results

Table 26 Results from Lightrun tube sequencing at Eurofins GATC services. Though mutant F.n.n. pilA1:his sequencing results confirmed the presence of Francisella, these bacteria were exposed to contamination, and was lost from further analysis.

***Francisella* mutants sorted with flowcytometry for verification**	**Template used for PCR**	**Primers used**	**Bacteria is *Francisella noatunensis* subsp. *noatunensis***
F.n.n. pilA6:his	DNA extract	F+R	Yes
F.n.n. ΔpilD pilA6:his	DNA extract	F	Yes
F.n.n. ΔpilD pilA1:his	DNA extract	F	No results
F.n.n. pilA6:his	Colony PCR	F+R	No results
F.n.n. pilA6:his	Colony PCR	F	Yes
F.n.n. ΔpilD pilA6:his	Colony PCR	F+R	Yes
F.n.n. ΔpilD pilA6:his	Colony PCR	F	Yes
F.n.n. ΔpilD pilA1:his	Colony PCR	F+R	Yes
F.n.n. clpB	Colony PCR	F+R	Yes
F.n.n. ΔpilD pilA6:his	DNA	R	Inconclusive
F.n.n. ΔpilD pilA1:his	DNA	R	Inconclusive

Francisella sp. strain EPZJ1811 16S ribosomal RNA gene, partial sequence

Sequence ID: MK757443.1 **Length:** 1526 **Number of Matches:** 1

Range 1: 1047 to 1338 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
512 bits(277)	4e-141	287/292(98%)	0/292(0%)	Plus/Minus

```
Query  35    TACTAACGATTCCGACTTCATGCAGTCGAGTTGCAGACTGCAATCCGGACTAAGAGTACC  94
             ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1338  TACTAGCGATTCCGACTTCATGCAGTCGAGTTGCAGACTGCAATCCGGACTAAGAGTACC  1279

Query  95    TTTCTGAGTTTCGCTCCAGCTCGCACCTTCCCAGCCCTCTGTAATACCCATTGTACCACG  154
             ||||||||||||||||||||||||||||| |||||||||||||||||||||||| ||||
Sbjct  1278  TTTCTGAGTTTCGCTCCAGCTCGCACCTTCGCAGCCCTCTGTAATACCCATTGTAGCACG  1219

Query  155   TGTGTAGCCCTGATCGTAAGGGCCATGATGACTTGACGTCGTCCCCACCTTCCTCCGCCT  214
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1218  TGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGACGTCGTCCCCACCTTCCTCCGCCT  1159

Query  215   TGTCAGCGGCAGTCTCAATACAGTACCCAACTTAATGATGGTAACTATCAATAGGGGTTG  274
             |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1158  TGTCAGCGGCAGTCTCAATAGAGTACCCAACTTAATGATGGTAACTATCAATAGGGGTTG  1099

Query  275   CGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCC  326
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1098  CGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCC  1047
```

Figure 21- Sequence retrieved from eurofins-GATC were inserted in nucleotide BLAST tool from NCBI. Figure shows the most significant alignment. Results shows that bacterial strains is highly likely to be Francisella.

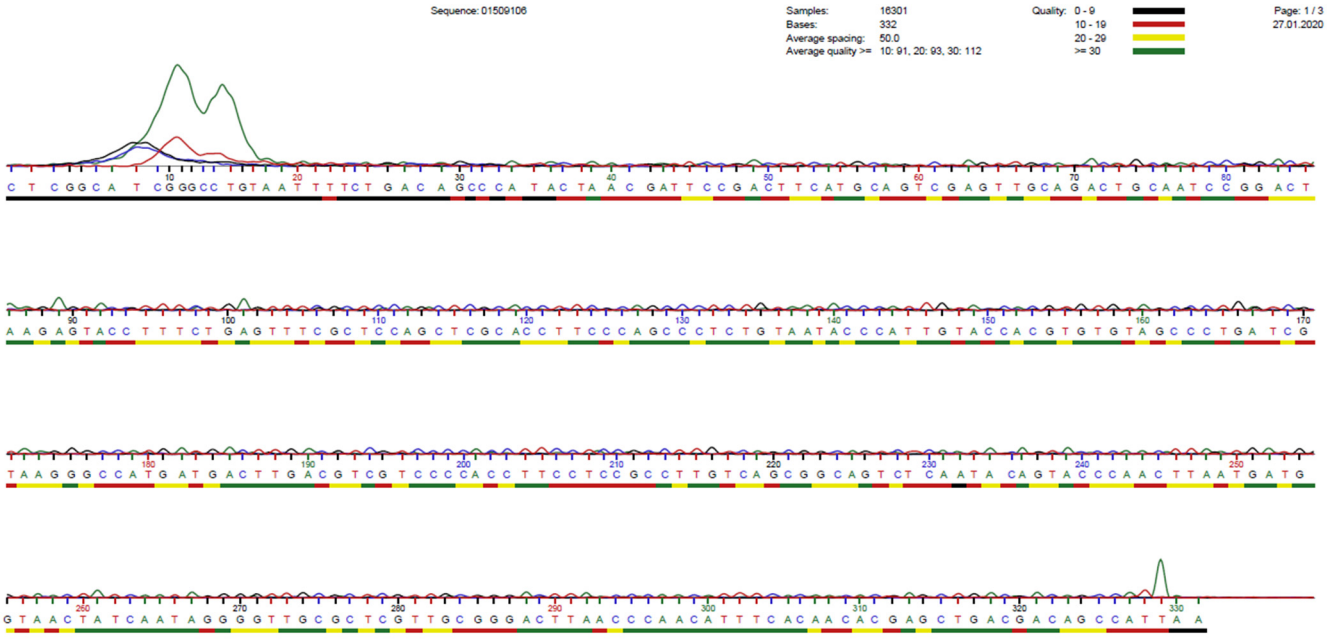

Figure 20 Base calling results retrieved from GATC from F.n.n. pilA6:his mutant. Results are not optimal, but sufficient for this experiments purpose. Colors indicate the quality of base calling (color codes can be found in upper right corner).

Appendix 6: Averaged normalized expression of immune genes in Atlantic salmon

The following tables contains the average normalized expression of the respective immune genes in both kidney and spleen of Atlantic salmons. The normalized expression is averaged towards the average gene expression in the control group (saline injected).

Table 27 Mean +/- SD, biological samples (n) and p-value of genes in spleen at time point 0 and 4 WPC.

		Untreated			Adjuvant			40 µg MV+adjuvant			100 µg MV		
Gene	WPC	Mean±SD	n	P	Mean±SD	n	P	Mean±SD	n	P	Mean±SD	n	P
SOCS	0	1.99±1.59	4	0.32	1.10±0.34	6	0.99	1.49±0.62	4	0.84	1.77±1.36	4	0.54
MPEG1	0	0.76±0.24	4	0.95	1.54±0.99	6	0.42	1.13±0.20	4	0.99	0.80±0.70	4	0.97
MHCIIB	0	1.52±0.21	4	0.35	0.93±.035	6	1.00	1.73±0.76	4	0.12	1.52±0.78	4	0.37
IL-1β	0	0.46±0.43	4	0.70	0.73±0.69	6	0.95	0.72±.061	4	0.96	0.27±0.14	4	0.46
IL-8	0	2.67±0.60	4	>0.99	17.64±17.77	6	0.11	17.65±21.43	4	0.17	4.79±2.17	4	0.97
IL-12	0	1.01±0.17	4	>0.99	0.89±0.39	6	>0.99	2.87±1.55	4	**0.01****	0.45±0.28	4	0.71
IL-10	0	0.28±0.33	4	0.98	2.30±4.16	6	0.80	2.05±2.76	4	0.92	0.15±0.09	4	0.96
SOCS	4	3.07±2.11	4	**0.02***	1.09±0.61	6	>0.99	1.49±0.74	5	0.86	2.01±0.80	4	0.39
MPEG1	4	1.38±0.52	4	0.78	1.56±0.15	6	0.41	2.36±0.96	5	**0.01****	1.52±0.95	4	0.56
MHCIIB	4	1.56±.0.33	4	0.16	1.00±0.23	6	>0.99	0.84±0.34	5	0.93	1.73±0.55	4	**0.04***
IL-1β	4	1.74±0.47	4	0.11	0.79±0.27	6	0.89	1.14±0.97	5	0.98	0.97±0.05	4	>0.99
IL-8	4	2.42±1.08	4	**0.02***	1.72±0.54	6	0.26	1.14±0.31	5	0.99	2.43±1.12	4	**0.02***
IL-12	4	1.25±0.75	4	0.89	0.53±0.08	6	0.38	0.56±0.22	5	0.48	1.65±0.41	4	0.21
IL-10	4	1.48±2.50	4	0.96	0.27±0.33	6	0.80	1.11±1.63	5	>0.99	0.29±0.18	4	0.86

Table 28 Mean +/- SD, biological samples (n) and p-value of genes in Liver at time point 0 and 4 WPC.

		Untreated			Adjuvant			40 µg MV+adjuvant			100 µg MV		
Gene	WPC	Mean±SD	n	P	Mean±SD	n	P	Mean±SD	n	P	Mean±SD	n	P
SOCS	0	1.43±1.14	4	0.79	0.55±0.60	6	0.72	0.43±0.18	5	0.58	1.47±0.49	3	0.80
MPEG1	0	0.15±0.12	4	0.18	0.31±0.28	6	0.25	0.25±0.15	5	0.22	0.09±0.07	3	0.19
MHCIIB	0	1.57±1.27	4	0.80	0.72±0.60	6	0.97	1.31±1.30	5	0.96	1.23±0.39	3	0.99
IL-1β	0	0.02±0.02	4	0.42	0.07±0.13	6	0.39	0.05±0.09	5	0.40	0.02±0.01	3	0.50
IL-8	0	6.99±12.30	4	0.93	11.3±13.83	6	0.61	12.16±17.57	5	0.58	16.67±24.00	3	0.42
IL-12	0	0.04±0.04	4	0.43	0.18±0.41	6	0.48	0.03±0.01	5	0.37	0.03±0.01	3	0.49
IL-10	0	0.01±0.01	4	0.45	0.28±0.58	6	0.62	0.02±0.03	5	0.40	0.02±0.01	3	0.53
SOCS	4	2.65±0.90	4	0.09	1.15±1.01	6	>0.99	1.46±0.66	6	0.88	2.20±2.01	4	0.29
MPEG1	4	0.67±0.32	4	0.92	1.86±1.18	6	0.23	1.16±0.42	6	0.95	0.30±0.27	4	0.49
MHCIIB	4	2.10±0.96	4	0.18	1.60±0.81	6	0.58	1.40±0.47	6	0.84	1.68±.086	4	0.57
IL-1β	4	3.20±3.66	4	0.83	3.66±7.33	6	0.64	2.77±2.36	6	0.87	1.18±0.93	4	>0.99
IL-8	4	2.86±2.62	4	0.26	1.60±1.12	6	0.92	1.55±0.85	6	0.94	2.52±2.52	4	0.42
IL-12	4	1.97±1.31	4	0.22	0.83±0.45	6	0.99	2.65±1.10	6	**0.02***	2.31±0.97	4	0.12
IL-10	4	0.53±0.49	4	0.99	2.13±3.91	6	0.82	1.74±1.69	6	0.95	0.29±0.24	4	0.97

Table 29 Mean +/- SD, biological samples (n) and p-value of genes in kidney at time point 0 and 4 WPC.

		Untreated			Adjuvant			40 µg MV+adjuvant			100 µg MV		
Gene	WPC	Mean±SD	n	P	Mean±SD	n	P	Mean±SD	n	P	Mean±SD	n	P
SOCS	0	3.30±1.38	4	**<0.01****	1.02±0.67	6	>0.99	1.13±0.48	5	>0.99	2.54±0.62	4	0.03*
MPEG1	0	0.82±0.22	4	0.98	1.33±1.22	6	0.82	0.98±0.20	5	>0.99	1.83±0.32	4	0.22
MHCIIB	0	1.56±0.24	4	0.19	0.98±0.72	6	>0.99	1.12±0.31	5	0.98	2.07±0.26	4	**<0.01****
IL-1β	0	1.22±0.61	4	>0.99	1.58±2.10	6	0.87	1.34±0.98	5	0.98	0.87±0.28	4	>0.99
IL-8	0	2.77±1.32	4	0.73	2.73±1.06	6	0.68	6.28±5.13	5	**0.02***	4.79±2.67	4	0.15
IL-12	0	1.08±0.67	4	>0.99	1.11±1.66	6	>0.99	1.72±1.52	5	0.78	0.59±0.16	4	0.97
IL-10	0	0.69±0.68	4	>0.99	3.89±7.62	6	0.64	3.67±3.29	5	0.72	0.32±0.11	4	>0.99
SOCS	4	2.19±0.73	4	0.21	1.22±0.85	6	0.98	2.28±1.30	6	0.10	1.43±0.67	4	0.90
MPEG1	4	2.46±0.61	4	**0.01****	1.22±0.46	6	0.96	1.39±0.65	6	0.78	1.66±0.86	4	0.44
MHCIIB	4	1.72±0.33	4	**0.01****	1.16±0.12	6	0.82	1.14±0.29	6	0.86	1.76±0.42	4	**<0.01****
IL-1β	4	1.89±0.35	4	0.28	1.03±0.38	6	>0.99	1.87±1.29	6	0.22	1.31±0.74	4	0.94
IL-8	4	2.74±1.03	4	0.08	1.93±0.74	6	0.43	1.79±0.53	6	0.57	2.46±2.36	4	0.16
IL-12	4	1.34±0.23	4	0.85	0.95±0.30	6	>0.99	1.34±0.20	6	0.79	2.62±1.52	4	**<0.01****
IL-10	4	1.66±2.20	4	0.96	0.66±1.03	6	>0.99	2.55±2.66	6	0.45	0.37±0.15	4	0.97