A new vaccine strategy to combat viral infections in European-farmed finfish

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Doctoral thesis 2019

PhD programme in **Advanced Immunology**

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Thesis submitted as partial fulfilment of the requirements for the degree of

Doctor of Advanced Immunology
Universitat Autònoma de Barcelona

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ABSTRACT

The aquaculture industry is a sector of dynamic growth, providing a high quality food source in the context of diminishing wild fish stocks and the need for global food security. Viral diseases are a major threat to finfish production and therapeutics are still lacking. Typically, commercially available vaccines are in the format of inactivated virus and are targeted to only a few, high market value fish species. The production and delivery process is expensive, with individual administration via injection. In this scenario, developing new, effective, practical vaccines which could be suitable for mass vaccination is a priority in the industry.

In this thesis we have drawn on recent work in biomaterials science to seek innovative strategies. We have nanostructured antigenic fish viral proteins as bacterial inclusion bodies (IBs), produced in *Escherichia coli*. The attractiveness of IBs as a vaccine design for aquaculture is that they are cheap, safe and stable *in vivo* without encapsulation, in contrast to soluble proteins. They provide a reservoir of biologically active protein which is slowly released. Here we target three viruses relevant in European finfish farming: The emergent reassortant strain of viral nervous necrosis virus (VNNV strain RGNNV/SJNNV) affecting Mediterranean marine farmed fish, and infectious pancreatic necrosis virus (IPNV) and viral haemorrhagic septicaemia virus (VHSV), both affecting salmonids. We present a comprehensive study of the production and immune response to three protein nanoparticle IBs made of antigenic proteins from each virus, VNNV coat protein C, IPNV capsid protein VP2 and a VHSV glycoprotein G fragment.

We demonstrate the three nanoparticles are taken up by zebrafish liver cells (ZFL) using flow cytometry and confocal microscopy. Using qPCR, we show the nanoparticles have immunostimulant properties *in vitro*, evoking an anti-viral innate immune response in ZFL and primary trout macrophage cultures. We also demonstrate by oral gavage that zebrafish can take up the nanoparticles through the intestine as a proof of concept for oral delivery. No toxic effects were observed *in vivo* or in an MTT cytotoxicity assay *in vitro*.

In *in vivo* farmed fish models using Senegalese sole (*S. senegalensis*) and rainbow trout (*O. mykiss*), we report the nanostructured viral proteins VNNV-C^{NP} and VHSV-G-frg16^{NP}, when injected intraperitoneally (i.p.), can raise specific, antiviral antibodies, as a surrogate of protection. Moreover, we show the anti-VHSV IgMs raised in trout are neutralizing, and upon infectious challenge with the virus, survival of vaccinated fish is consistent with protection. In Senegalese sole we also performed immune gene expression studies and compared the antibody response for i.p. and oral delivery using a novel oral intubation method. Our findings show the oral route can raise anti-viral antibodies but needs to be optimized to avoid a tolerance response.

We conclude this new approach to develop practical vaccines for farmed fish holds promise.

RESUM

Una nova estratègia de vacunació per combatre les infeccions víriques en espècies aqüícoles d'interès comercial

La indústria aqüícola és un sector en creixement dinàmic i una font d'aliment d'alta qualitat en un context de decreixement dels estocs d'espècies salvatges i de necessitat d'assolir una seguretat alimentaria global. Les malalties víriques són encara una gran amenaça per a la producció de peixos i manquen estratègies terapèutiques antivirals. Normalment, les vacunes disponibles al mercat són en format de virus inactivats i només es dirigeixen a unes poques espècies de peixos amb un alt valor comercial. D'altra banda el procés de producció i administració de vacunes és car ja que requereix una administració individualitzada per injecció. En aquest escenari el desenvolupament de noves vacunes, eficaces i pràctiques que siguin adequades per a la vacunació massiva és una prioritat per a la indústria.

En aquesta tesi hem abordat el problema utilitzant nous biomaterials per buscar estratègies innovadores. Hem produït de forma nanostructurada proteïnes virals antigèniques com a cossos d'inclusió bacterians (CI), produïts en *Escherichia coli*. El principal atractiu dels CI com a vacunes per a l'aqüicultura, és que són barats, segurs i estables *in vivo* sense necessitat d'encapsulació, a diferència de les proteïnes recombinants solubles. Proporcionen un reservori de proteïnes biològicament actives que s'alliberen lentament. En aquesta tesi les hem produït de tres virus rellevants per l'aqüicultura europea: la soca emergent del virus de la necrosi nerviosa (VNNV, RGNNV/SJNNV) que afecta els peixos de conreu marins mediterranis i els virus de la necrosis pancreàtica viral (IPNV) i de la septicèmia hemorràgica viral (VHSV), els quals afecten a salmònids. Presentem una caracterització completa de la producció i la resposta immune a tres nanopartícules fetes de proteïnes virals antigèniques de cada un dels virus: la proteïna C del VNNV, la proteïna VP2 de la càpside del IPNV i un fragment de la glicoproteïna G del VHSV.

Demostrem que les tres nanopartícules són internalitzades per les cèl·lules hepàtiques del peix zebra (ZFL) utilitzant citometria de flux i microscòpia confocal. Utilitzant qPCR, demostrem que les nanopartícules tenen propietats immunostimulants *in vitro*, evocant una resposta immunitària innata anti-viral tant en ZFL com en cultius primaris de macròfags de truita. També demostrem que el peix zebra pot internalitzar les nanopartícules a través de l'epiteli de l'intestí el que suposa una prova de concepte per l'administració oral de les vacunes. En cap cas es van observar efectes tòxics *in vivo* ni en els assajos de citotoxicitat *in vitro*.

En dos models *in vivo* utilitzant llenguado senegalès (*S. senegalensis*) i truita irisada (*O. mykiss*), demostrem que les proteïnes virals nanoestructurades VNNV-C^{NP} i VHSV-G-$frg16^{NP}$, quan s'injecten per via intraperitoneal (i.p.), poden generar anticossos antivirals específics que ens indiquen que hem induït protecció. A més, mostrem que les IgMs anti-VHSV que es produeixen

en truita tenen capacitat neutralitzant i que, després d'una infecció amb el virus, la supervivència dels peixos vacunats és coherent amb la protecció observada. Amb el llenguado senegalès es van realitzar també estudis d'expressió gènica i es va comparar la resposta d'anticossos després d'una administració i.p. i una administració oral utilitzant un nou mètode d'intubació. Els nostres resultats mostren que la via oral pot generar anticossos anti-virals, però ha de ser optimitzada per evitar una resposta de tolerància. Concloem que aquest nou enfoc per produir vacunes per espècies de peixos d'interès comercial desenvolupat en el marc d'aquesta tesis doctoral, mostra resultats molt prometedors.

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ABBREVIATIONS

AM Affinity maturation

ANOVA Analysis of variance

APC Antigen presenting cell

BSA Bovine serum albumin

CCL4 C-C motif chemokine ligand 4

CD Cluster of differentiation

CMI Cell-mediated immunity

CMV Cytomegalovirus

CpG 5'-cytosine-phosphate-guanine-3'

Cq Quantification cycle

CTL Cytotoxic T lymphocyte

CyHV-3 or (**KHV**) Cyprinid herpes virus or (Koi herpes virus)

d.p.a. Days post administration

d.p.i. Days post infection

DC Dendritic cell

DMSO Dimethyl sulphoxide

EDTA Ethylenediaminetetraacetic acid

ELISA Enzyme-linked immunosorbent assay

EPC *Epithelioma papulosum cyprini* cell line

EU European Union

FAO Food & Agriculture Organization of the United Nations

FBS Fetal bovine serum

FDA United States Food & Drug Administration

FESEM Field emission scanning electron microscopy

FFU Focus forming units

FITC Fluorescein isothiocyanate

G Glycoprotein

GALT Gut-associated lymphoid tissue

GFP Green fluorescent protein

GMO Genetically modified organism

i.m. Intramuscular

i.p. Intraperitoneal

IB Bacterial inclusion body

IEL Intraepithelial lymphocyte compartment

IFN Interferon

Ig Immunoglobulin

iNOS Inducible nitiric oxide synthase

IHNV Infectious haematopoietic necrosis virus

IL Interleukin

IPNV Infectious pancreatic necrosis virus

IPTG Isopropyl β-D-1-thiogalactopyranoside

IRF Interferon regulatory factor

iRFP Infra-red fluorescent protein

ISAV Infectious salmon anaemia virus

ISG Interferon stimulated gene

IWV Inactivated whole virus

LCDV Lymphocystis disease virus

LPS Lipopolysaccharide

MALT Mucosa-associated lymphoid tissue

MFI Mean fluorescence intensity

MHC Major histocompatibility complex

MS-222 Tricaine mesylate

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NCBI National Center for Biotechnology Information

NK Natural killer

NOD Nucleotide-binding & oligomerization domain

NP nanopellet

O/N Overnight

OD Optical density

OIE World Organization for Animal Health

ORF Open reading frame

PAMP Pathogen molecular pattern

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PGN Peptidoglycan

PLGA Poly (lactic-co-glycolic acid)

ABBREVIATIONS cont.

Poly (I:C) Polyinosinic:polycytidylic acid

POMV Pilchard orthomyxovirus

PRR Pattern recognition receptor

qPCR Quantitative real-time PCR

RAG Recombinant activating genes

RGD Arginine-glycine-aspartic acid

RGNNV Red-spotted grouper nervous necrosis virus

RIG Retinoic acid-inducible gene

RLR RIG-I-like receptor

RPS Relative percentage survival

RSIV Red sea bream iridovirus

RT Room temperature

RT-HKM Rainbow trout head kidney macrophages

SAV Salmonid alphavirus

SD Standard deviation

SEM Standard error the mean

SJNNV Striped jack nervous necrosis virus

STAT Signal transducer & activator of transcription

SVCV Spring viremia of carp virus

$TCID_{50}$ Median tissue culture infectious dose

TCR T cell receptor

TGF Transforming growth factor

Th Helper T lymphocyte

TLR Toll-like receptor

TMB 3,3'5,5' tetramethylbenzine

TNF Transforming necrosis factor

Treg Regulatory T cell

UK United Kingdom

USA United States of America

UV Ultraviolet

VER Viral encephalopathy & retinopathy

VHSV Viral haemorrhagic septicaemia virus

VLP virus-like particle

VNNV Viral nervous necrosis virus

VP Viral protein

ZFL zebrafish liver cell line

INTRODUCTION

Metal sculpture of tuna,
Costa de la luz, Càdiz.
Photo: R Thwaite

1. Aquaculture: a highly relevant sector in food production

Global awareness of fish and shellfish as key contributors to food security and nutrition is growing. Food fish (finfish, crustaceans and molluscs) are a primary source of protein and essential nutrients. In 2015 food fish represented approximately 17% of animal protein consumed worldwide and greater than 50% in some countries (1). Fish are very efficient converters of feed into food in terms of biomass, compared to beef and pork, and in addition to protein, they provide a source of long-chain, polyunsaturated fatty acids (LC-PUFAS), vitamins D, A, B and minerals. Fish are therefore highly relevant in food security and health strategies (2). During the last three decades, with stocks of wild fish in decline, aquaculture production has increased at an average annual growth rate of more than 8 % (2). Meanwhile, captured production has remained at around 90 million tonnes / year. (**Figure 1**). With increased demand and improved technology, the Food and Agriculture Organization of the United Nations (FAO) predicts by 2030 aquaculture will be producing around 109 million tonnes, accounting for ~54% of the world's food fish production (1).

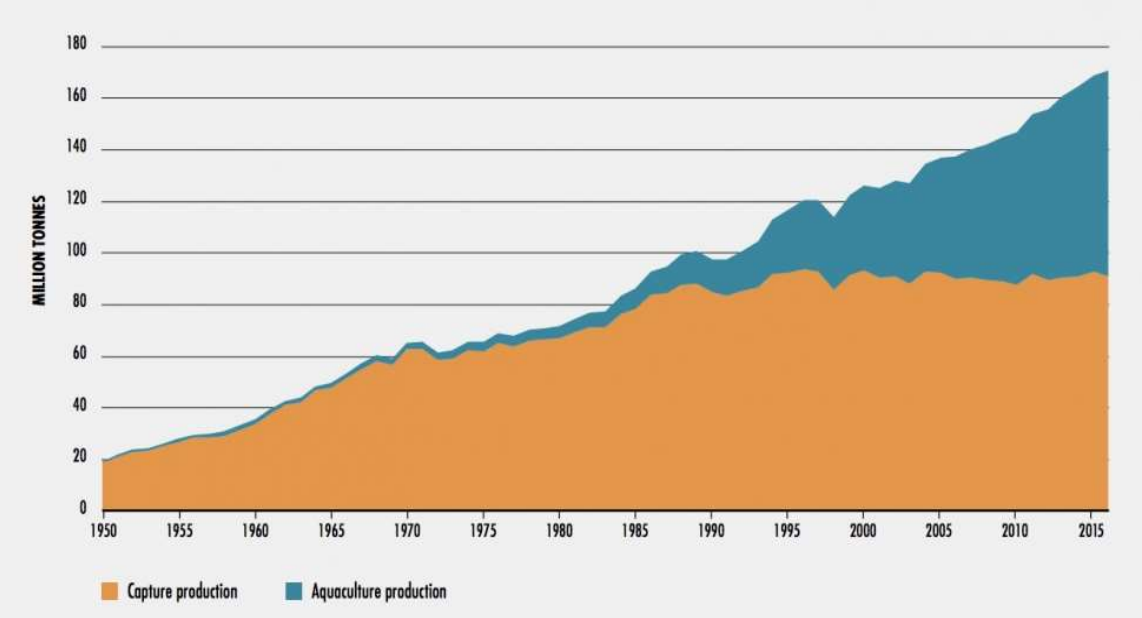

FIGURE 1: World food fish production in millions of tonnes from 1950-2015 from ■ captured fisheries & ■ aquaculture. Food fish includes finfish, crustacea & molluscs. Reproduced from FAO report 2018 (1)

In Europe (not including Norway) aquaculture production is predominantly finfish (~80%) and molluscs (~20%). The amount of food fish production has actually been decreasing, but the value / kg has increased due to more Atlantic salmon production. The European Union (EU) imports 60% of its fish food supply to meet demand (3). There are 5 main producing countries within the

EU: Spain, France, Italy, Greece and the UK, currently providing employment for over 90,000 people (1). In the EU, 90% of all finfish production focuses on 5 species: rainbow trout (*Oncorhynchus mykiss),* Atlantic salmon (*Salmo salar*), gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*) and common carp (*Cyprinus carpio*) (3). Spain however stands out for its diversification of the industry, producing turbot (*Scophthalmus maximus*), Senegalese sole (*Solea senegalensis*) and meagre (*Argyrosomus regius*) among others, as well as sea bream, sea bass and trout. The most common method of production is using offshore marine cage systems, since recirculated aquaculture systems are costly, reducing competitiveness (3). It is interesting to note that the FAO considers availability of space as a major limiting factor in the expansion of sustainable aquaculture, as well as the threats of climate change and disease (1).

2. Viral diseases in finfish aquaculture

The aquaculture environment provides favourable opportunities for pathogens to propagate. High density water rearing brings many fish together causing chronic stress and an increased probability of transmission, facilitating the development of clinical disease. In addition, the use of open-net cages off shore for marine fish or in rivers and lakes for inland aquaculture means that farmed and wild animals are in the same water column and diseases carried by wild fish at low density may cause outbreaks in intensively reared farmed fish (4). While bacteria are the most common cause of infectious disease in aquaculture (5) and parasites such as sea lice (*Lepeophtheirus salmonis*) and the salmon fluke (*Gyrodactylus salaris)* have had severe effects on the salmon industry (6-8), viruses have caused a greater negative impact, in general, since they are more difficult to control. Young fish and larvae are highly susceptible, many viral diseases still lack commercial vaccines or therapeutics and more knowledge of resistance and pathogenesis is needed (5). International trade of live aquatic animals and their products also contributes to the emergence of viruses, with geographical redistribution favouring disease development in the same or other fish species (4) Nevertheless, the rapidly advancing field of viral metagenomics is providing tools that can vastly improve identification, epidemiological monitoring and study of pathogens, including emerging viruses (9). This should permit better surveillance and quicker action for disease control.

There are a plethora of fish viruses among veterinary aquatic pathogens, reviewed in Kibenge and Godoy's (2016) comprehensive book Aquaculture Virology (10). Here we have compiled a table showing general characteristics of the main viruses affecting European finfish farming.

TABLE 1: Characteristics of the main viruses affecting European finfish farming; adapted with permission from (11) including information from (10) and viral zone https://viralzone.expasy.org/ (ds = double stranded, ss = single stranded)

Virus Family	Genus	Virion size (nm), shape	Genome size (kb)	DNA/RNA, segments	Virus species, acronymn	Typical host	Temper-ature °C	Associated pathology	Ref
Alloherpesviridae	*Cyprinivirus*	200, enveloped, spherical	295	dsDNA, linear	Cyprinid herpesvirus 3 (CyHV-3) (KHV)	common carp	18 -28	gill inflammation and necrosis,	(12)
Iridoviridae	*Lymphocystis-virus*	200-230, enveloped, spherical	102	dsDNA, linear	Lymphocystis disease virus 1 (LCDV-1)	sea bream, flounder	20 - 25	nodular skin lesions	(13)
Birnaviridae	*Aquabirnavirus*	60, non-enveloped spherical	5.9	dsRNA 2	Infectious pancreatic necrosis virus (IPNV)	salmon, trout, turbot	10 -15+	haemorrhage in pancreas, necrosis in liver, pancreas	(14)
Nodaviridae	*Betanodavirus*	33, non-enveloped, icosahedral	4.5	+ ssRNA 2	Nervous necrosis virus (NNV)	sea bass & bream, sole, turbot	20-30	encephalopathy & retinopathy (VER), vacuoles brain, retina	(15)
Orthomyxoviridae	*Isavirus*	90-140, enveloped, pleomorphic	14.3	- ssRNA 8	Infectious salmon anemia virus (ISAV)	salmon	10+	anorexia, lethargy, anaemia, haemorrhages	(16)
Rhabdoviridae	*Novirhabdovirus*	110 x 70, enveloped, bullet	11	- ssRNA 1	Infectious haemato-poietic necrosis virus (IHNV)	trout, salmon	12-15	anaemia, necrosis of kidney, spleen	(17)
"	*Novirhabdovirus*	200 x 75, enveloped, bullet	11.2	- ssRNA 1	Viral haemorrhagic septicaemia virus (VHSV)	trout, turbot	3-15	anorexia, internal haemorrhaging	(18)
"	*Vesiculovirus*	80-180 x 60-90, enveloped, bullet	11	- ssRNA 1	Spring viremia of carp virus (SVCV)	common carp	10-17	peritonitis, haemorrhages, enlarged kidney, spleen	(19)
Togaviridae	*Alphavirus*	70, enveloped, spherical	11.8	+ssRNA 1	Salmonid alphavirus (SAV)	salmon, trout	10+	anorexia, necrosis in pancreas, heart muscle, sleeping disease (SAV2)	(20)

Among the ten top significant fish diseases listed by the World Organization for Animal Health (OIE), http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2019/ seven are caused by viruses listed in **Table 1**. (CyHV-3 (KHV), ISAV, IHNV, epizootic IHNV, VHSV, SVCV and SAV). This clearly demonstrates the relevance and pathogenicity of these viruses. The other OIE listed diseases are caused by the water mould (*Aphanomyces invadens*) in the Asia-Pacific, the salmon fluke (*G. salaris)* and by red sea bream iridovirus (RSIV) which severely affects cultured fish in Japan. No bacterial diseases are OIE listed. One reason is that conventional vaccines from inactivated whole bacteria are quite cheap to produce, as bacteria can usually be cultured easily in broth media (21). This, and the use of antibiotics, has provided more tools to combat bacterial diseases. We now give an overview of the teleost immune system, before discussing strategies in vaccinology to control the spread of fish viral diseases.

3. The teleost immune system

Teleosts, are a large, highly diverse clade of ray-finned fish representing approximately half of extant vertebrate species. They are found in almost all aquatic environments of different temperatures, salinity, oxygen concentration, water pressure and thus encounter a wide variety of pathogens (22). They are free-living organisms from early embryonic stages of life, initially relying on innate (non-specific) immune responses for survival. Fish genomes are particularly heterogenous due to cycles of whole genome duplications and differential losses (genome contraction) (23) (**Figure 2**).

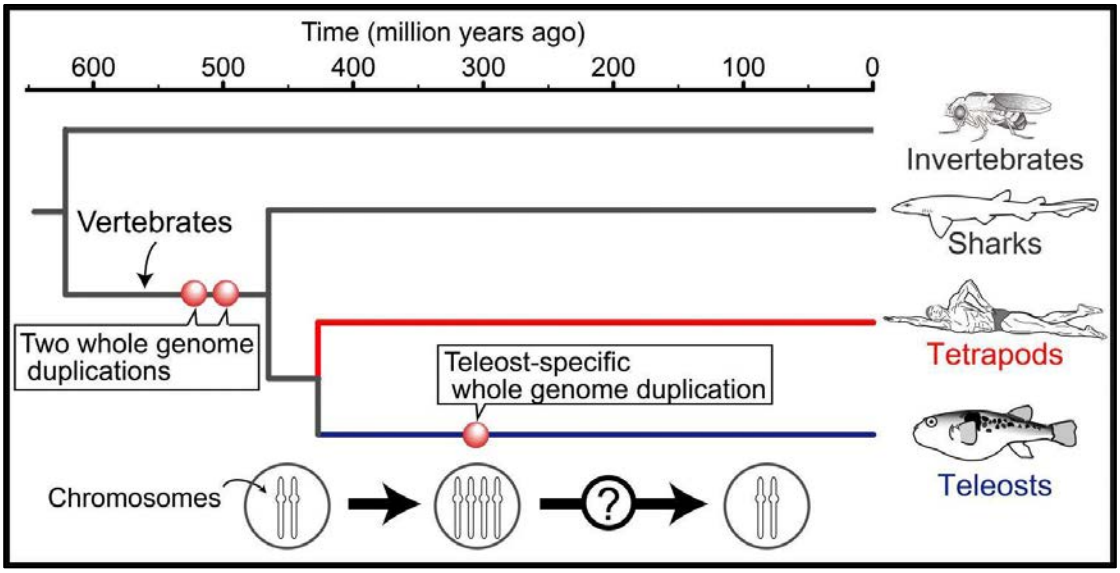

FIGURE 2: Whole genome duplication events along representative animal lineages. Reproduced from Okinawa Institute of Science and Technology Graduate University (OIST), press release https://www.oist.jp/news-center/press-releases/mysteries-bony-fish-genome-evolution Related publication (23)

The role of the immune system is primarily to recognize and eliminate foreign threats and maintain homeostasis. The two main components are the innate system and the adaptive immune system. The innate system is an initial barrier to pathogen spread. It senses intruders and provides a rapid non-specific response to clear them. Innate immunity is the only defence system in invertebrates and is fundamental in vertebrates. In fish this response includes anti-bacterial peptides in skin secretions, lysozyme, lectins, and the complement system (24). It also includes several germ-line encoded receptors known as pattern recognition receptors (PRRs). These are nucleotide-binding and oligomerization domain (NOD)-like receptors (25), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and toll-like receptors (TLRs) (26). The PRRs recognize pathogen molecular patterns (PAMPs) such as lipopolysaccharides (LPS), peptidoglycan (PGN) and viral nucleic acids (27) reviewed in (28). When PAMP ligands bind to PRRs intracellular signalling is triggered, inducing immune effector molecules via cytokines. Cytokines are immune modulators of an inflammatory response which recruits cells to the site of pathogen intrusion, activates antimicrobial effectors and, in vertebrates, stimulates adaptive immunity. Fish have all the key pro-inflammatory cytokines such as interleukin-1β (IL-1β), TNF-α, IL-6 and downstream effectors, as well as classical anti-inflammatory cytokines IL-10 and TGFβ to downregulate and resolve a response to regain homeostasis (29). Further, the innate immune system stimulates T and B cells and antigen presenting cells through the activation of phagocytes, complement and cell receptors, as well as via cytokines and chemokines (30, 31). The innate and adaptive systems are therefore networked.

The adaptive immune system is a specific, clonal response to antigen. It is considered to have arisen in jawed fish approximately 500 million years ago with the advent of the recombinant activating genes (RAG). This evolutionary step provided the possibility for rearrangement of immunoglobulin super family genes, B-cell and T-cell receptors and Major Histocompatibility complex (MHC) classes, creating a potential repertoire for recognition of antigenic epitopes virtually beyond limit (30). Cartilaginous fish (sharks in **Figure 2**) are the earliest living organisms with a primitive adaptive immune system. They have immunoglobulins, T cell antigen receptors, major histocompatibility complex (MHC) class I and II molecules, spleen and thymus (32). Teleosts have some additional features. They lack lymph nodes and bone marrow, but haematopoiesis takes place in the head kidney which is the functional ortholog of mammalian bone marrow. Teleosts do not have germinal centres. They lack antibody class-switch recombination and have less diversity of immunoglobulins than mammals (32). Indeed, the fish adaptive immune system is less complex than in higher vertebrates, while the innate system has a wider range of molecules and plays a crucial role starting from embryonic stages. **Table 2** gives an overview of similarities and differences between the immune systems of teleosts and mammals.

TABLE 2: Comparative immune system of teleosts & mammals; adapted with permission from (5) including information from (33-35)

Feature in immune system	Teleosts	Mammals
Physical barriers & interfaces	Skin mucus; scales; gills	Skin; respiratory epithelium of the lungs
Immune effector cell types	Neutrophil; Eosinophil; Monocyte/Macrophage; Dendritic cell (only identified in trout gill structures and zebrafish so far); NK-like cells; T lymphocytes (helper, cytotoxic); B lymphocytes (plasma cells)	Neutrophil; Eosinophil; Basophil; Mast cell Monocyte/Macrophage; Dendritic cell; NK cell; T lymphocytes (helper, cytotoxic); B lymphocytes (plasma cells)
Major antigen presentation cells	Monocyte/macrophage	Dendritic cell, macrophage/monocyte
Lymphoid tissues	Head kidney; Thymus; Spleen; Gut-associated lymphoid tissue (not well organized); Interbranchial lymphoid tissue; skin- & nasopharynx-associated lymphoid tissue	Bone marrow; Thymus; Spleen; Lymph nodes; Gut-associated lymphoid tissue; Germinal centres
Antibodies: Immunoglobulin (Ig) diversity	IgM; IgD; IgT	IgM; IgD; IgA; IgE; IgG
Antibody response to challenge/rechallenge	Slow and weak memory response (temperature dependent)	Fast and strong memory
Affinity maturation (AM)	Low affinity, and low AM	High affinity and high AM

As regards T cells, **Table 2** shows teleosts have T cell populations generally similar to those found in mammals. Teleosts have two major T cell receptors (TCR), TCRαβ and TCRγδ and $CD8^+$, $CD4^+$ cell subsets related to cytotoxic and helper T (Th) lymphocyte functions, including subtypes Th1, Th2 and Th17 (36). Vaccines which induce both cellular mediated immune (CMI) responses and humoral (antibody) responses are considered the most effective (37), as will be discussed later in this chapter. We now give some more detail about aspects of the teleost immune response, referring particularly to viral infection in sections 3.1. and 3.2.

3.1. Antiviral innate immunity in teleosts

A key cytokine network in response to viruses is the interferon (IFN) system. Sensing of viral intrusion by PRRs leads to the activation of interferon signalling pathways. Interferons are master antiviral cytokines, inducing many genes known as interferon -stimulated genes (ISGs), which

perform diverse antiviral effector and regulatory functions. An overview of the IFN signalling pathway and its effects is shown **Figure 3** (38). The pathway includes Janus and tyrosine kinases (JAK, TYK); transcription factors such as interferon regulatory factors (IRFs) and signal transducer and activator of transcription (STATs)(39); as well as evolutionarily conserved antiviral proteins such as Mx, which can interact with viral capsid and nucleoproteins, presumably disrupting localization and protein complex association with viral RNA-dependent RNA polymerase, inhibiting transcription (40). Another conserved antiviral ISG is viperin which acts against a broad range of viruses using various mechanisms such as blocking viral particle release, interacting with host proteins needed for viral replication and perhaps even modulating innate antiviral signalling (41). Upregulation of interferons or of downstream ISGs can be used as an indicator of activation of an innate antiviral response.

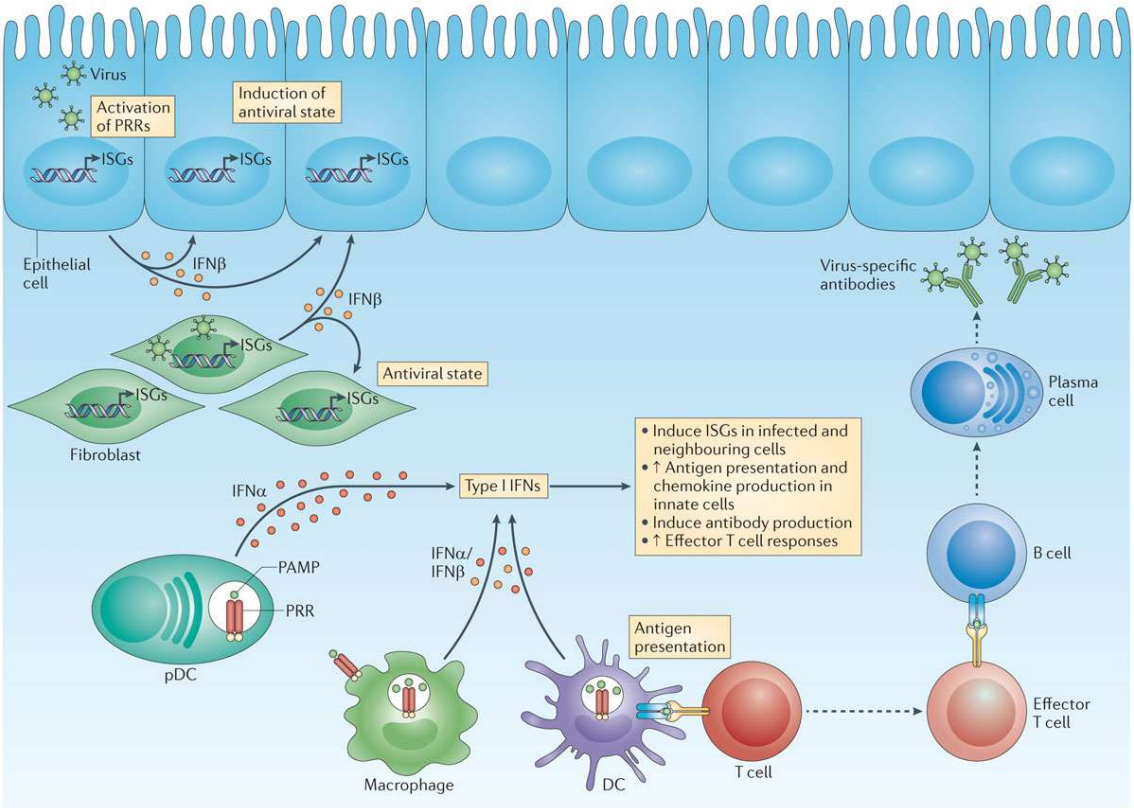

FIGURE 3: Type 1 interferon (IFN) signalling involved in innate & adaptive immunity. Pathogen is sensed through pattern-recognition receptors (PRRs) on innate immune cells (macrophages & dendritic cells (DCs)) on the plasma membrane, in endosomes & in the cytosol. Type 1 IFNs are secreted, inducing the expression of IFN- stimulated genes (ISGs) and antiviral effector molecules are produced. Innate immune cells also respond to type I IFNs by enhancing antigen presentation & production of immune response mediators, such as cytokines & chemokines. In addition, adaptive immunity is affected. Type I IFNs can augment antibody production by B cells & amplify the effector function of T cells. PAMP, pathogen-associated molecular pattern; pDC, plasmatoid DC. Reprinted with permission from (38).

3.2 Teleost B cells and viral immunity

Apart from the production of specific antibodies via the adaptive immune response, teleost B cells can perform a number of functions which suggests they play a role in the immediate response to infection. They are able to directly sense pathogens as they constitutively express PRRs (42); they have a potent phagocytic capacity leading to degradative pathways (43) and they can express antimicrobial peptides (44), traits resembling macrophage activities. These functions are not present in conventional mammalian B2 cells. The majority of B cells found in teleosts are IgM^+ IgD^+ B cells, which are postulated to resemble innate B cell subsets in mammals, noting that fish rely on IgM responses without class switch recombination or germinal centres and affinity maturation is poor (45). In acute viral infections in fish, specific IgMs are produced later than when mortalities occur. Thus, innate, rapid responses are critical. Fish also have natural antibodies in their sera. These are immunoglobulins present prior to pathogen encounter, produced by B cells in homeostasis and providing a first line of defence before the antibody response is mounted (46). In trout cells natural antibodies have been demonstrated to protect against VHSV or IPNV infection *in vitro* (47). Natural antibodies, along with the whole range of innate responses, could therefore contribute to protecting fish in early stages of viral infection, before a specific response is mounted (45).

3.3 Mucosal immunity

Fish are continuously exposed to microbes in the water circulating around them. Fish are immersed in a microbially very rich environment compared to terrestrial animals, posing a greater challenge to their epithelial barriers (35). Their mucosa is a frontline where microbes can invade, colonize and establish infections. Consequently, fish have protective mucus and epithelial barriers coupled to mucosa-associated lymphoid tissues (MALT) which sense and react to danger signals. Teleosts have four such tissues: gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT). Sensing of pathogens via the MALT triggers an immediate innate immune response. Then mucosal B and T lymphocytes may be activated to induce cytotoxic T lymphocyte (CTL) responses and antibody secretion (35). Note however the mucosal system is a tolerogenic environment, since many of the antigens presented, particularly the GALT, are from harmless commensal bacteria or food. Viral or other antigens in mucosal vaccines can only be effective if the antigens can reach inductive sites and stimulate strong immunity, thus overriding tolerance (48). Mucosal organs have antigen presenting cells (APCs) that uptake, process and present antigens to naïve B and T lymphocytes, which given the appropriate environment (for

example pro-inflammatory) will lead to adaptive and cellular immune responses. T cells in the intraepithelial lymphocyte compartment (IEL) are predominantly CD8+, while CD4+ T cells are more common in the lamina propia (49). There is an enriched population of IgT^+ B cells in the teleost mucosa, which is a completely separate B cell lineage. IgT (also called IgZ) is considered a specialized mucosal antibody and the ratio IgT/ IgM is 63 fold higher in gut mucus than in serum (50). IgT is phylogenetically distinct from IgA in mammals, but they are both predominantly secreted mucosal antibodies, transported by the polymeric Ig receptor (pIgR) (49), and have functions in common such as the coating of gut luminal bacteria. It is thought that IgT is involved in gut homeostasis and may have an anti-inflammatory role; as does IgA (50). **Figure 4** shows a schematic representation of immune cells found in the teleost intestine (49).

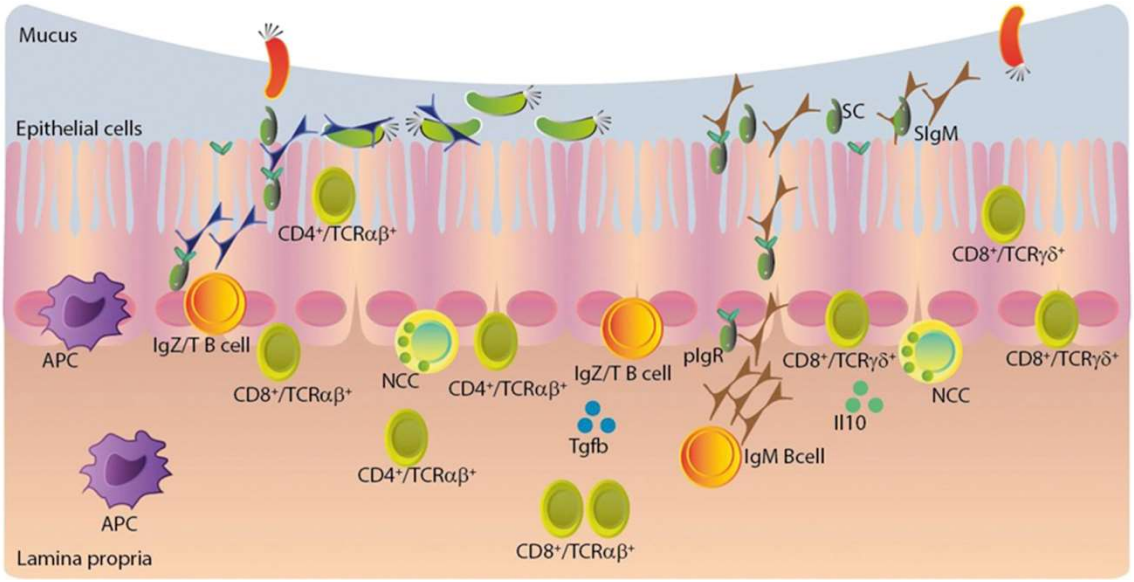

FIGURE 4: Different immune cells in the teleost intestine; reprinted with permission from (49). CD8α+ TCRαβ are more numerous than CD4+ TCRαβ. Most TCRγδ are CD8+. B cells in the intraepithelial lymphocyte compartment (IEL) are mainly IgZ/T+. IgM+ B cells are in connective tissue. Non-specifc cytotoxic cells (NCC) may be found in the IEL. Also pictured: Antigen presenting cells (APC), pathogenic microbes (red), commensal microbes (green). IgT blue, IgM brown, polymeric immunoglobulin receptor (pIgR)-secretory component (SC) dark green. Cytokines related to oral tolerance induction TGFβ blue dots and IL10 green dots.

4. Current strategies in fish vaccinology

Vaccination is a very effective way of protecting fish from viral and bacterial diseases, especially under the high-density growth conditions found in finfish farming. The use of vaccines has also had a positive impact on the reduction of antibiotic use, for instance in Norwegian salmon farming (51). Management strategies such as increased disease surveillance, better farm biosecurity protocols and the use of immunostimulants and probiotics also contribute to improved disease control (52). Vaccine development against fish parasites such as sea lice is also on the agenda

(53). But if we consider what is a good vaccine for fish farming, the efficiency of protection is not the only factor to be taken into account. Rather we also need to consider what is a feasible vaccine for development in the context of disease severity and prevalence, the target species and its production as food. There are safety considerations, practical considerations and cost/benefit issues summarized in **Figure 5**. To translate research into practice it is necessary to keep these factors in mind.

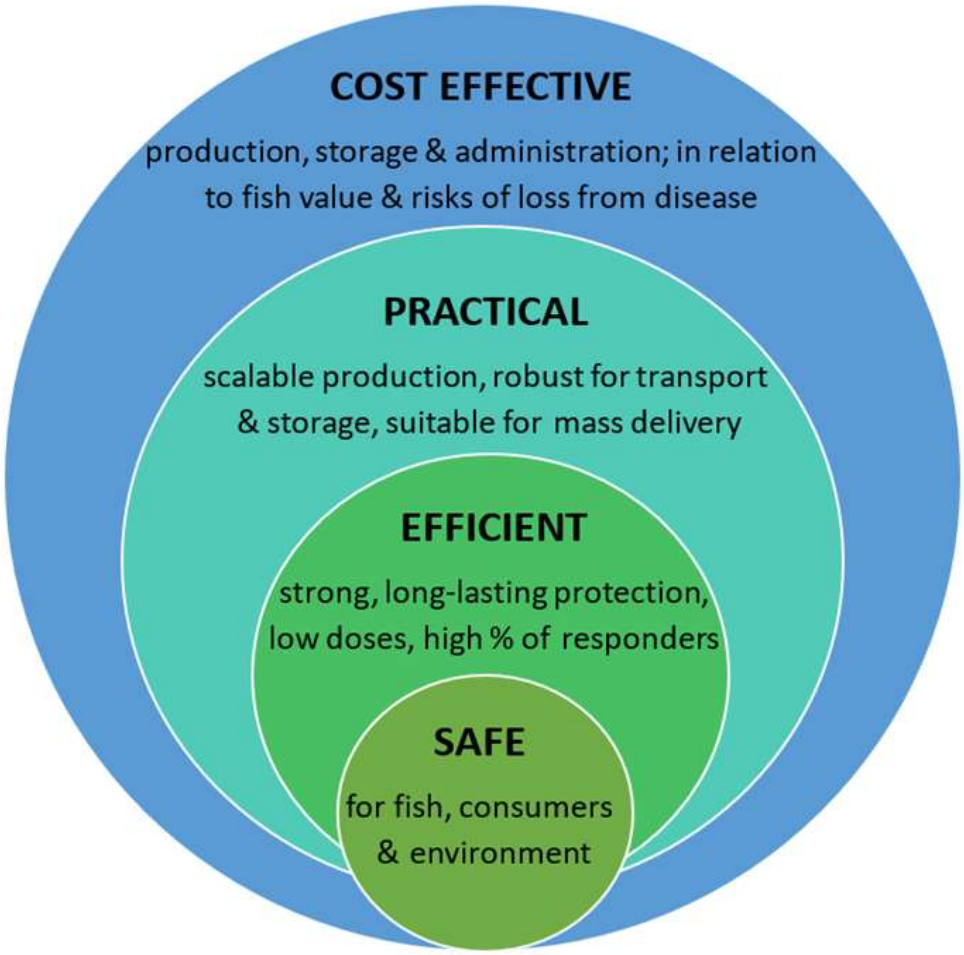

FIGURE 5: Features to consider in vaccine design. A feasible vaccine strategy involves many factors, beginning with safety. The concept of fish value is not restricted to market value. A fish such as tilapia can be very valuable in terms of food security in low & middle-income countries.

4.1 Vaccination delivery routes

In terms of practicality and effectiveness, a fundamental aspect the researcher needs to consider is the administration route of the vaccine. Primarily there are 3 delivery routes used: injection - intraperitoneal (i.p.) or intramuscular (i.m.), bath immersion and oral administration in the feed. The nasal route is also being explored in trout (54). **Table 3** shows the key characteristics for the three main administration routes currently used. Each method has advantages and disadvantages.

TABLE 3: Characteristics of main vaccine administration routes used in aquaculture; based on (51, 52, 55)

Route	Equipment & labour	Fish size	Amount vaccine / fish	Mass vaccination feasible	Stressful for fish	Strong protection	Common Use
INJECTION intraperitoneal (i.p.) water-in-oil based vaccines, intramuscular (i.m.) DNA vaccines	EXTENSIVE anaesthetic, automated syringes, individual delivery, highly labour intensive	medium-large NOT feasible for fry & small juvenile fish	low	NO Unless using automated vaccination machines e.g. in Norway's salmon industry https://www.skalamaskon.no/	VERY Individual handling, fish may be injured, operators also	YES Direct delivery of antigen to immune system, strong humoral response. Easy to combine with adjuvant	Majority of vaccines are injectable. Many more are available for bacterial than viral diseases. Both mono & multi-valent vaccines are used.
IMMERSION dip or bath, in vaccine suspension	MODERATE baths, labour to set up baths & transfer fish	fry & juveniles NOT feasible for larger fish	high	YES But limited by bath and fish size	MODERATE transfer and crowding in bath	VARIABLE Induces mucosal immune response, humoral response not as strong	Useful for diseases occurring at fry stage or to combine with an injected/ oral boost when fish are larger
ORAL mixed or coated on feed	MINIMAL	ALL life stages, once fish is immune-competent	medium	YES Multiple administrations easy. Can be used in ponds & cages which is impossible for other routes. Amount eaten by each fish can vary	ZERO vaccine included in feeding regime	VARIABLE Antigen must survive gastric degradation & induce protection in tolerogenic gut environment	Ideal method for practicality, but few commercial vaccines available. Most used as booster.

For example, an injected vaccine can be delivered in a small concentrated dose with adjuvant, and generally provides strong protection. In fact, the majority of fish vaccines are injected (56). However, logistically and cost-wise injection is a major operation and not possible for small fish. Moreover, it is stressful for fish and can cause injury and melanisation at the injection site (52). A way of balancing protection and the logistics of each delivery method is to perform vaccination regimes with alternating delivery routes and multiple antigens. For example, for European sea bass (*D. labrax*) a regime used against vibriosis (*Lactobacillus anguillarum*) and pasteurellosis (*Photobacterium damselae*) is as follows. When fish are 1 g, a combined water-based immersion vaccine is used, which is repeated when fish reach 5 g weight. After this, fish are given a final water-in-oil injected vaccine when they are 15 g minimum size (51). There is a general consensus that the oral route is ideal as regards ease of antigen delivery, unrestricted fish size (although fish must be old enough to be immunocompetent), absence of stress and wide applicability in different rearing scenarios. Nevertheless, few commercial vaccines are available. Current efforts focus on encapsulation methods and adjuvants so that antigens can withstand degradation in the gut, be taken up and induce an effective immune response locally and systemically via the GALT (55).

4.1.1 Oral tolerance

Indeed, the main challenges posed by using the oral route are gastric degradation of the antigen and the tolerogenic gut environment. Different encapsulation methods to protect and deliver vaccines will be discussed in section 4.2.6. Here, the phenomenon of oral tolerance will be briefly described, and further discussion is found in chapter two. Oral tolerance is considered the "default immune pathway" in the mammalian gut and is a hypo-responsiveness to fed antigens (57). Considering many of the antigens in this environment come from the gut flora or food, it is not surprising that regulatory mechanisms exist to control immune responses towards innocuous elements. On the other hand, the GALT, like other MALTs, plays a critical role in protection against pathogens and both cellular and humoral immune responses can be activated. Oral antigens are taken up by enterocytes in the gut epithelium or sampled by dendritic cells (DCs) (in fish these are macrophage-like cells) extending into the lumen. In mammals, M cells can also take up antigen from the lumen. A relevant factor in tolerance mechanisms is the amount of antigen fed. In mammals, low doses favour the induction of regulatory T cells (Tregs); while high doses induce anergy or deletion (57). The frequency of feeding is also influential, as is the production of anti-inflammatory cytokines TGFβ and IL-10, which induce and sustain tolerogenic Tregs and DCs (58). In fish, tolerance has been manifested by a decrease in serum antibodies following each of two oral boosts of unencapsulated inactivated IPNV in Atlantic salmon (59). The boosts were given one year post an i.p. injected oil-adjuvant based vaccine. The decrease in antibodies was

accompanied by significant upregulation of genes *foxP3*, *tgfβ* and *il-10*. However, in the same paper, a single oral boost with alginate-encapsulated inactivated IPNV increased the systemic immune response, but a double boost did not. This demonstrates that protection initiated via oral delivery is possible, but the right formulation and dose regime is required. This is an active area of research (55).

4.2 Types of vaccines against fish viral diseases

Now focusing on viruses, we present the main different vaccine types either commercially available or being developed experimentally. A list of commercial vaccines and their characteristics is provided in **Table 4**. Vaccine encapsulation strategies and adjuvants will also be discussed.

4.2.1 Live attenuated vaccines

Live vaccines are attenuated viruses that are genetically modified or cultured to disable virulence, or they are recombinant antigens encoded in viral vectors. The attenuated virus replicates in host cells at a much lower rate than wildtype and its pathogenicity is curbed. The great advantage of live vaccines is they can induce both cellular and humoral immune responses. They gain entry into the cells through binding to surface receptors like the native virus and then replicate in the cytosol using the host cell machinery. Intracellular processed antigens are presented on the cell surface by MHC-I molecules to interact with CD8+ T cells and activate cell-mediated immunity. Replicating virus triggers the innate immune response and when secreted, APCs engulf exogenous viral antigens and present them on MHC-II to then interact with CD4+ helper T cells, inducing humoral responses (58, 60). The result is a high level of protection. A number of successful studies on experimental live vaccines have been published particularly against VHSV and IHNV (See (60) Table 2). However, the major handicap of live vaccines is the possible reversion to virulence. This has been reported, for example, with an attenuated strain of IPNV after infected Atlantic salmon juveniles were subject to stress (61). Other risks are residual virulence and spread from vaccinated to naïve subjects. This risk has prevented licensing of live attenuated vaccines. An exception is the KV-3 (KoVax Ltd. Israel) against Koi Herpes virus (KHV or CyH-3) (52), developed through serial passaging and UV irradiation. Currently licensed for use in Israel it nevertheless has residual virulence for fish weighing less than 50 g and was withdrawn from use in the USA (12). The onus now is to develop attenuated vaccines via rational, controlled methods of genetic modification, such as deletion of genes encoding virulence factors, so that reversal to virulence can be excluded (62).

4.2.2 Inactivated whole virus (IWV) vaccines

Wild type virus killed by physical or chemical methods such as heat or formalin was the initial strategy used to produce fish vaccines. These vaccines retain the virus's exposed surface antigens and inactivated genome. As such, they induce a strong humoral response, being taken up by the exogenous route, followed by presentation to CD4+ cells via the MHC-II pathway, leading to production of specific antibodies (60). The vaccine is typically delivered by i.p. injection with an adjuvant and protection via antibodies is usually high. A limitation is that culturing the virus is costly and not possible for all viruses, for instance lymphocystis virus (52). Nevertheless, the majority of commercial vaccines available are of this type (see Table 3 in (52)). IWV vaccines are available against RSIV, SAV, ISAV and IPNV, produced by a range of companies with licensing in Japan, Norway, UK, Chile and Canada. Experimental vaccines have been reported against at least 8 other viruses (see Table 5 in (60)). In 2018, Pharmaq released the first European commercial vaccine against Nodavirus (RGNNV genotype) in sea bass (*D. labrax*), called ALPHA JECT micro® 1Noda, with marketing authorization for Spain, Italy, Croatia and Greece. It is an inactivated virus in emulsion (mineral oil adjuvant) for i.p. injection to fish of minimum weight 12 g. This demonstrates that the aquaculture sector is continuing to develop IWV vaccines.

4.2.3 DNA vaccines

The principle of DNA vaccination is to inject plasmid DNA encoding an immunogenic protein into the muscle (i.m.). The plasmid is introduced into host cells and the transcribed antigens replicate in the cytosol leading to MHC-I presentation. Secreted protein is phagocytized by APCs, leading to MHC-II presentation. The antigenic protein is produced in a natural host and therefore folding, glycosylation and display of epitopes is likely to follow the pattern of the native virus (60). All these factors contribute to DNA vaccines' potential to induce strong protection. This has been particularly evident with fish rhabdoviruses (63). DNA vaccines have been designed to produce the immunogenic G glycoprotein, which in rhabdoviruses protrudes as multiple spikes on the virus surface. The first DNA vaccine for aquaculture, APEX-IHN (Novartis/Elanco), was licensed in 2005 in Canada to protect Atlantic salmon against the novirhabdovirus IHNV (64). However, it was not until 2017 that a DNA vaccine was authorized for marketing in Europe. CLYNAV (Elanco) is a polyprotein-encoding DNA vaccine against Salmon Pancreas Disease Virus (SPDV), an alphavirus. Meanwhile, there are many experimental DNA vaccines under development; Collins (2019) Table 1, lists 18 vaccines, excluding novirhabdoviruses (65).

An advantage of DNA vaccines is that the antigen can be modified or additional features can be incorporated such as genetically encoded adjuvants, the so called molecular adjuvants (66). These are signalling molecules or cytokines added to the plasmid to increase immunogenicity and

efficiency. A very successful example can be found in Atlantic salmon in which a DNA vaccine against ISA was co-injected i.m. with a plasmid encoding IFN1 (a, b or c). The joint treatment raised higher antibody titres and the relative percentage survival (RPS) after an ISAV challenge increased from 43% (only DNA vaccine) to 91% (DNA vaccine plus IFN1 molecular adjuvant). Treating with the IFN1 plasmid alone gave and RPS of only 9% (67). Given the promise of a number of experimental DNA vaccines, it may appear surprising that so few have been licensed. The main obstacle is safety concerns regarding the integration of foreign DNA, which typically includes a human viral promoter, CMV, into an animal used for human consumption. Alternatives with hybrid promoters that include fish sequences have been tested (68). But there is also the risk of the plasmid carrying an antibiotic resistance gene being released in to the environment. Finally, the issue of whether the vaccine and the recipient are considered genetically modified organisms (GMOs) is a concern (65).

4.2.4. Subunit vaccines

Subunit vaccines are composed of specific viral proteins produced in a recombinant protein expression system. They may contain single or multiple antigens and can be produced in bulk in biotechnological systems and then purified. The attractiveness of this approach is scalable, cheap production and safety, as there is no replicating virus or DNA introduced to the fish. Results have been variable. The challenge has been to avoid rapid degradation of the protein in processing, storage and within the animal (52). We will discuss encapsulation techniques that can protect antigens from such degradation in section 4.2.6. Sufficient immunogenicity has also been an issue. Nevertheless, some subunit vaccines have been very effective and are commercialized. Virbac-Centrovet have produced recombinant ISAV hemagglutinin esterase in yeast (*Saccharomyces cerevisiae*) as an orally delivered subunit vaccine against ISAV for Atlantic salmon, licensed in Chile. MSD animal health manufactures a subunit vaccine AQUAVAC®IPN Oral containing viral proteins VP2 and VP3 from IPNV produced in yeast (*Pichia pastoris),* licensed in Canada. It is delivered in the feed to protect Atlantic and Coho salmon, and rainbow trout from infectious pancreatic necrosis. Experimental vaccines include other expression systems such as *Escherichia coli* which has been recently used to produce an antigenic viral structural protein, VP35, of grass carp reovirus (GCRV), achieving moderate protection (60% RPS) when injected i.p. in grass carp against grass carp haemorrhagic disease (69).

TABLE 4: Commercially available vaccines against viral diseases in finfish aquaculture (4 pages)

Virus	Vaccine type	Antigen	Delivery route	Product name	Target species	Producer	Country with license to use
DNA viruses							
Iridovirus	Inactivated whole virus (IWV)	virus	Injection i.p.	AQUAVAC®IridoV	tilapia, barramundi	MSD Animal Health	Singapore
KHV (CyHv-3)	live attenuated	virus	immersion/ injection	KV-3	common carp	Ko Vax Ltd, Israel	Israel
Red sea bream iridovirus	IWV	virus	injection i.p.	Killed iridovirus vaccine	Red sea bream, striped jack (*Seriola* genus)	The Research Foundation for Microbial Diseases of Osaka University Japan	Japan
RNA viruses							
IHNV	DNA	Recombinant G protein	injection i.m.	Apex-IHN	salmonids	Elanco Canada Ltd	Canada
IPNV	subunit	VP2 & VP3 capsid proteins	oral	AQUAVAC®IPN Oral	salmonids	MSD Animal Health	Chile
IPNV	Micro matrix encapsulated IWV	2 strains of IPNV	oral	Blueguard®IPN Oral	salmonids	Centrovet	Chile
IPNV	IWV	3 strains of IPNV	immersion	Blueguard®IPN Inmersión	salmonids	Centrovet	Chile
IPNV	IWV	2 strains of IPNV	injection i.p.	Blueguard®IPN inyectable	salmonids	Centrovet	Chile

Virus	Vaccine type	Antigen	Delivery route	Product name	Target species	Producer	Country with license to use
RNA viruses cont.							
IPNV	IWV	virus (1 strain)	injection i.p.	ALPHA JECT®1000	Atlantic salmon, rainbow trout	Pharmaq	Chile
Bivalent: IPNV & *A. salmonicida*	IWV + inactivated bacteria	virus & bacteria	Injection i.p.	ALPHA JECT®2-2	Atlantic salmon	Pharmaq	UK
Multivalent: IPNV & various combinations of bacteria	IWV + inactivated bacteria	virus & bacteria (1-4 species)	injection i.p.	ALPHA JECT®IPNV-Flavo 0,025/micro 2/micro 3/ 4-1	Atlantic salmon	Pharmaq	Chile
Multivalent: IPNV & 4 bacteria	subunit & inactivated bacteria	VP2 capsid & bacteria	injection i.p.	NORVAX®Minova 6	Atlantic salmon	MSD Animal Health	Norway
Multivalent: IPNV & 5 bacteria	IWV + inactivated bacteria	virus & bacteria	injection i.p.	Alpha Ject 6-2	Atlantic salmon	Pharmaq	Norway, The Faroe Islands
Multivalent: IPNV & 5 bacteria	IWV + inactivated bacteria	virus & bacteria	Injection i.p.	Pentium Forte Plus	Atlantic Salmon	Elanco Canada Ltd (export only)	Norway
Multivalent: IPNV & 2 or 3 bacteria	IWV + inactivated bacteria	virus (2 strains) & bacteria	injection i.p.	Blueguard® SRS +IPN+VO/SRS+IPN+FU+ VO inyectable	Atlantic salmon	Centrovet	Chile
Bivalent: IPNV & *P. salmonis*	IWV + inactivated bacteria	virus & bacteria	injection i.p.	Birnagen Forte 2	Atlantic salmon	Elanco Canada Ltd	? export only, conditionally licensed

Virus	Vaccine type	Antigen	Delivery route	Product name	Target species	Producer	Country with license to use
RNA viruses cont.							
ISAV	Micro matrix encapsulated subunit	ISA proteins hemoglutinine & neuroaminidase	oral	Blueguard®ISA Oral	Atlantic salmon	Centrovet	Chile
Bivalent: ISAV & *P. salmonis*	Micro matrix encapsulated subunit & inactivated bacteria	ISA proteins hemoglutinine & neuroaminidase & bacteria	oral	Blueguard®SRS+ISA Oral	Atlantic salmon	Centrovet	Chile
Multivalent: IPNV+ISAV & 2 or 3 bacteria	Subunit ISAV + inactivated IPNV (IWV) & bacteria	ISA proteins hemoglutinine & neuroaminidase, virus (2 strains) & bacteria	injection i.p.	Blueguard® SRS+IPN+VO+ISA/ IPN+SRS+AS+VO+ISA inyectable	Atlantic salmon	Centrovet	Chile
Multivalent: ISAV & 4 bacteria	IWV + inactivated bacteria	virus & bacteria	injection i.p.	Forte VII	Atlantic salmon	Elanco Canada Ltd	Canada
NNV	IWV	RGNNV virus	injection i.p.	ALPHA JECT micro 1 Noda	European Sea bass	Pharmaq	Croatia, Greece, Italy, Spain
POMV	IWV	virus	Injection i.p.	Certovac	Atlantic salmon	Tasmanian salmonid growers' association	Australia
SAV	IWV	virus	Injection i.p.	ALPHA JECT micro®1 PD	Atlantic salmon	Pharmaq	Ireland, Norway, UK
SAV	IWV	virus	Injection i.p.	Norvax® Compact PD	Atlantic salmon	MSD Animal Health	Chile, Norway, UK

Virus	Vaccine type	Antigen	Delivery route	Product name	Target species	Producer	Country with license to use
RNA viruses cont.							
SAV3	DNA	Salmon pancreas disease virus polyprotein	Injection i.m.	Clynav	Atlantic salmon	Elanco Canada Ltd (export only)	European Union, Norway
Trivalent: SAV, IPNV & *A. salmonicida*	IWV + inactivated bacteria	virus & bacteria	Injection i.p.	AquaVac PD3	Atlantic salmon	MSD Animal Health	UK, Ireland
Multivalent: SAV, IPNV & 5 bacteria	IWV + inactivated bacteria	virus & bacteria	Injection i.p.	AquaVac PD7	Atlantic salmon	MSD Animal Health	Norway

Sources:

Dhar, 2014 (52) and websites accesssed 18.4.2019

http://www.inspection.gc.ca/active/netapp/veterinarybio-bioveterinaire/vetbioe.aspx#table-heading

http://www.frdc.com.au/Media-and-Publications/FISH/FISH-Vol-26-4/Salmon-get-ready-for-their-flu-shots

https://www.merck-animal-health.com/species/aquaculture/salmon.aspx https://www.aquavac-vaccines.com/

https://www.centrovet.com/index.php/productos238/salmonicultura-peces

https://www.pharmaq.no/products/vaccination-of-fish/

4.2.5 Viral-like particle (VLP) and subviral particle (SVP) vaccines.

Viral structural proteins typically self-assemble to form viral capsids. This property has been taken advantage of to create another type of subunit vaccine, VLPs. These are made of recombinant viral capsid proteins that are allowed to spontaneously self-assemble. The resulting particles mimic the native virus in size and morphology, but do not contain genetic material. The host takes them up as a virus, but they are non-replicative and therefore, safe. When only some of the viral capsid proteins are present the protein structures are called SVPs (60). VLPs display viral epitopes as if they were a virus and this can lead to high protection. For example, convict grouper fish injected i.p. with VLPs of red-spotted grouper NNV produced in *S. cerevisae* elicited neutralizing antibodies and gave fish full protection against an RGNNV challenge. The same vaccine provided moderate protection when orally delivered (70). In another study, high antibody levels against NNV after injecting orange spotted grouper i.m. with NNV VLPs produced in *E. coli* were correlated with upregulation of IgM, MHC-II and CD4 (71), indicative of an immune response for antigens entering APCs via the extracellular route (60). The disadvantage in using VLPs is in the production, which requires isolation of highly purified protein and retrieval of the particles on a sucrose gradient after ultracentrifugation. A very interesting property is that VLPs could also be used as a vaccine carrier since they have a protein cage architecture. This is being explored with nodavirus (72). To date no VLP vaccines have been commercialized for fish, though viral capsid proteins of IPNV are being used in subunit vaccines.

4.2.6. Encapsulation techniques: nanocarriers, alginates and bio-encapsulation.

With particular relevance for oral vaccines, different methods of encapsulation have been developed to deliver antigens safely thorough the digestive tract and be taken up into the GALT. One mode is nanoencapsulation which involves preparing antigen loaded particles of diameter 1-1000 nm (73). In aquaculture two nanocarriers have been the most widely investigated: chitosan and poly (lactic-co-glycolic acid) or PLGA nanoparticles. Both polymeric nanoparticles are biocompatible, biodegradable and non-toxic which are fundamental features of safe drug delivery systems (74). They can penetrate the mucosa and are internalized into the host cell by endocytosis; **Figure 6**. This has been monitored for chitosan and PLGA with particle size of ~430 and ~300 nm respectively and the process was primarily clathrin mediated pinocytosis (75). Trafficking can vary with size and particle charge, which can be altered through functionalization, for instance with the cationic polymer poly-L-lysine (75). Another nanocarrier which readily enters fish cells is liposomes. Nanoliposomes of ~125 nm diameter carrying immunostimulants were shown to be internalized preferentially by caveolae-dependent endocytosis, but also by clathrin-mediated

endocytosis in zebrafish liver (ZFL) cell line and by macropinocytosis in trout macrophages (76), **Figure 6**. We now provide more information about these three nanocarriers.

Chitosan is a linear polysaccharide derived from the N-deacetylation of chitin, which can be cheaply prepared from shrimp shell waste (77) and has anti-microbial properties (78). Chitosan nanoparticles are formed by an ionic gelation mechanism and loading is achieved by dissolving the chitosan in 1 % acetic acid, then mixing and vortexing with an equal volume of the cargo material and allowing the particles formed to stabilize (79). Chitosan nanoparticles have a natural bio-adhesion to mucosal surfaces, demonstrated in fish (80), and have been used to orally deliver vitamin C to trout, evidencing *in vivo* controlled release and stimulation of the innate immune system (81). In a vaccine study, inactivated ISAV was loaded into chitosan nanoparticles which were delivered in the feed to juvenile Atlantic salmon. In addition, chitosan nanoparticles loaded with plasmid DNA coding for the replicase of alphavirus were included as a molecular adjuvant. Upon ISAV challenge the group fed only the viral loaded nanoparticle had an RPS of 40% but the group fed vaccine and adjuvant reached an RPS of 77% (82).

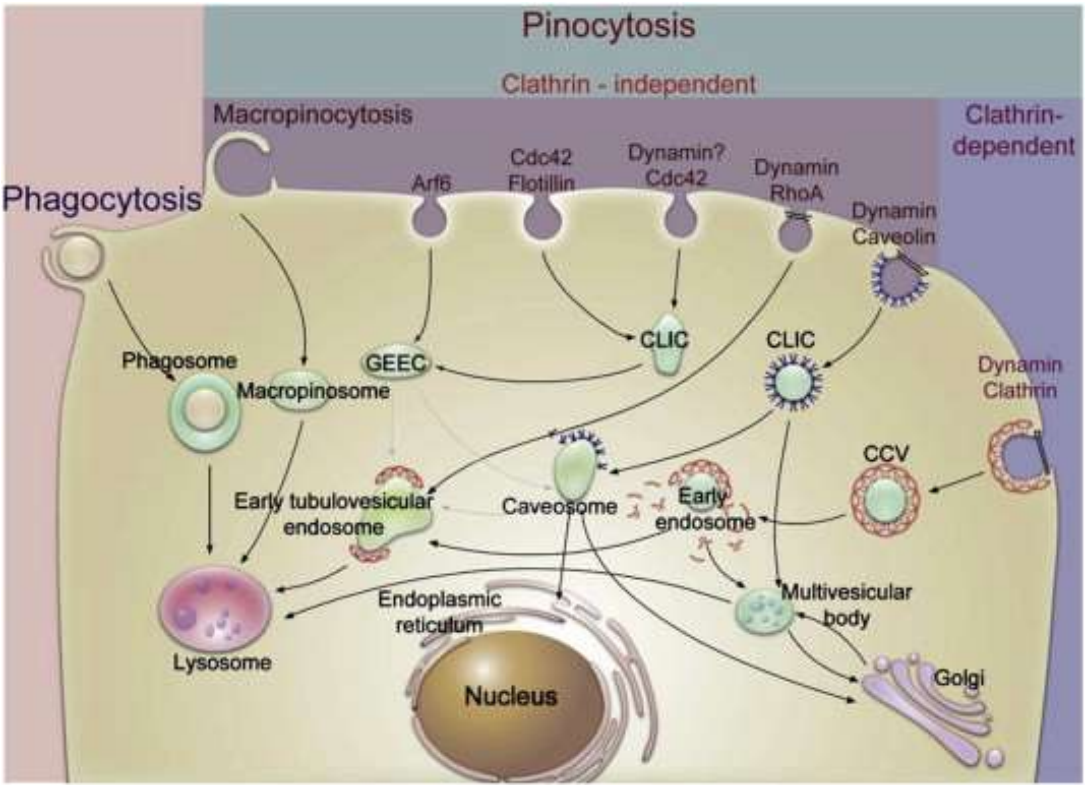

FIGURE 6: Different mechanisms of endocytosis, reprinted with permission from (75). Particles & solutes enter the cell via multiple pathways. Endocytosis occurs at portals of cellular entry in the plasma membrane, which engulfs the cargo into intracellular vesicles. Cargo is then sorted through endosomes or goes via the phagosome, depending on uptake. CCV = clathrin coated vesicles, CLIC = clathrin-independent carriers, GEEC = glycophosphatidylinositol-anchored protein-enriched compartment.

PLGA is a copolymer synthesized with lactic acid and glycolic acid monomers at an adjustable ratio. When the polymer is hydrolysed both monomers are released and metabolized through the citric acid cycle (83) PLGA has been approved for use in nanotechnology therapeutics by the United States Food and Drug Administration (FDA) (84). The nanoparticles are prepared by a single or double emulsion method in an organic solvent to which hydrophobic or emulsified hydrophilic cargo is added (85). Studies have shown that PLGA nanoparticles escape from the endo-lysosomal compartment into the cytosol after uptake (83). In fish gradual release of load has been shown after oral delivery and FITC-labeled PLGA nanoparticles could also be found in the blood (86). Experimental vaccines using PLGA encapsulated IWV or plasmid DNA in trout and salmon have shown low protection (60), but results for Japanese flounder, also called olive flounder (*Paralichthys olivaceus*) are very promising. PLGA nanoparticles loaded with a DNA vaccine encoding the major capsid protein of LCDV was administered to the stomach of young adult fish in suspension via a syringe. Post-delivery, RNA of the capsid protein was found in the gill, intestine, spleen and kidney, from 10-90 days, also specific antibodies were raised. Post-challenge, the LCVD infection rate was 16.7%, compared to 100% in fish vaccinated with naked plasmid or blank PLGA nanoparticles. These infected fish all presented characteristic nodular lesions on fins or skin (86). In another study, Kole, 2019, tested a formalin inactivated VHSV vaccine encapsulated in PLGA by both immersion and oral routes in olive flounder fingerlings. The administration regime was immersion followed two weeks later by either immersion or oral administration. Various immune genes including were upregulated in both systemic (head kidney) and mucosal (skin and intestine) immune compartments. Post VHSV challenge the RPS was 73.3% for the immersion/oral group and 60% for the immersion/ immersion group.

Nanoliposomes are self-sealing spheres made of phospholipid bilayers which can entrap hydrophilic drugs or incorporate lipophilic drugs into the bilayer. They have a large surface area to volume ratio and the composition is tuneable to achieve the properties desired such as targeting to cells or tissues and achieving controlled release of the cargo (87). An “in-feed” automated delivery platform was developed in Chile to mix and administer liposomal DNA vaccines (lipoplex) into fish feed. An IPNV DNA vaccine encoding for an immunogenic region of the viral capsid protein VP2 was tried in this system, fed in single or double dose. Following challenge with IPNV moderate to good protection was achieved, with the RPS of the single dose group being 58.2% and the double dose group 66% (88). In another oral vaccination study, common carp were fed with formalin-inactivated koi herpes virus loaded into liposomes and incorporated into food pellets. Orally vaccinated fish had significantly high neutralizing antibody titres against 2 different KHV (CyHV-3) isolates and following challenge, the RPS was 75% and 65% for each of the viral isolates (NKC03 and IKC03) respectively (89).

Another carrier which has been used widely in experimental vaccines for fish are alginate microspheres. Alginate is a polysaccharide copolymer of β-D-mannuronic acid and α-L-gluronic acid produced naturally in brown algae. These monomers are arranged in blocks in a chain and form a variable crosslinked matrix that can be used to trap drugs (90). Alginate microparticles are typically made by an external or internal gelation using an emulsification method (90) and the size varies with the formulation. Using the $CaCl_2$ production method microspheres are 10-100 μm or larger (58). They are muco-adhesive, with release occurring through degradation of the polymer network. This occurs at neutral or basic pH, while under acidic conditions the particles are more stable (91). Alginates are therefore very suitable for encapsulating fish oral vaccines. They resist the low pH in the fish stomach, which can reach pH 2 - 4, and then can release antigen in the fore or hind gut where the pH is neutral-basic. De las Heras, 2010, orally immunized fingerling trout (introducing a pipette tip to the mouth of anesthetized fish) with plasmid-loaded alginate microspheres, encoding the VP2 protein of IPNV. In vaccinated fish they detected innate immune responses, neutralizing antibody production and, after challenge, the RPS was 80% (92). A similar experiment was performed with a plasmid encoding the G protein of IHNV, in which anti-IHNV antibodies were produced and viral load was greatly reduced in tissues of vaccinated fish. But the RPS was not so high. It was significantly greater for the same DNA vaccine injected i.m. (70%) than the oral route (56%). Also, the oral route required 20-fold more plasmid DNA to get significant levels of IHNV transcripts in kidney and spleen (93). This is an example of the trade-offs between practical issues and the level of protection induced, which need to be considered when deciding the optimal delivery route.

Finally, a method of oral delivery which is gaining interest is bio-encapsulation. This involves transporting the vaccine in a live vector such as bacteria, yeast or invertebrates like the brine shrimp (*Artemia*), which are fish prey. Lactic acid bacteria are part of the fish gut microbiota and can survive passing through the upper gastrointestinal tract to then colonize the intestine. They are known to stimulate the innate immune system, promoting disease resistance, and are being developed as probiotics (94) and as oral vaccine carriers (95). Cui, 2015, used a genetically engineered *Lactobacillus* strain to surface-display both the G protein of SVCV and the ORF81 protein of KHV (96). The bacteria were mixed with raw fish feed components and the mixture was extruded into pellets that were fed to common carp and koi adults for 3 days, followed by 2 booster regimes. Significant levels of IgM were induced in vaccinated fish. After challenge with SVCV for common carp and with KHV for koi, viral load was greatly reduced. The RPS was 71% and 53% in vaccinated carp and koi respectively. Recently, Embregets 2019, demonstrated the potential of *Pichia pastoris* yeast as an oral antigen delivery vehicle for both adults and larvae. As a proof of concept, green fluorescent protein (GFP) was expressed in yeast, which was fed to

the planktonic crustacean *Daphnia,* the bio-encapsulator. Subsequently, the *Daphnia* were fed to flounder larvae. Cells positive for GFP were found in the larvae intestine (97).

4.2.7. Adjuvants

The purpose of adjuvants is to improve the immune response induced by an antigen in a vaccine formulation. Adjuvants vary widely in composition but can be broadly classified as immuno-enhancers or delivery systems (98). Immuno-enhancers or stimulants aid to activate pathways which induce innate immune responses, mainly targeting APCs. This increases the immune responses to the antigen co-delivered. For example, an adjuvant such as poly (I:C) can target intracellular receptors; **Figure 7** (99). Delivery systems can enhance the uptake of antigen by APCs and allow for slow release, delayed clearance and improved exposure to the immune system. They may also stimulate the innate immune system, chitosan being an example. We have presented various delivery systems used in fish vaccination in section 4.2.6. Here we give some examples of immune-enhancer adjuvants currently being used or developed in fish vaccinology.

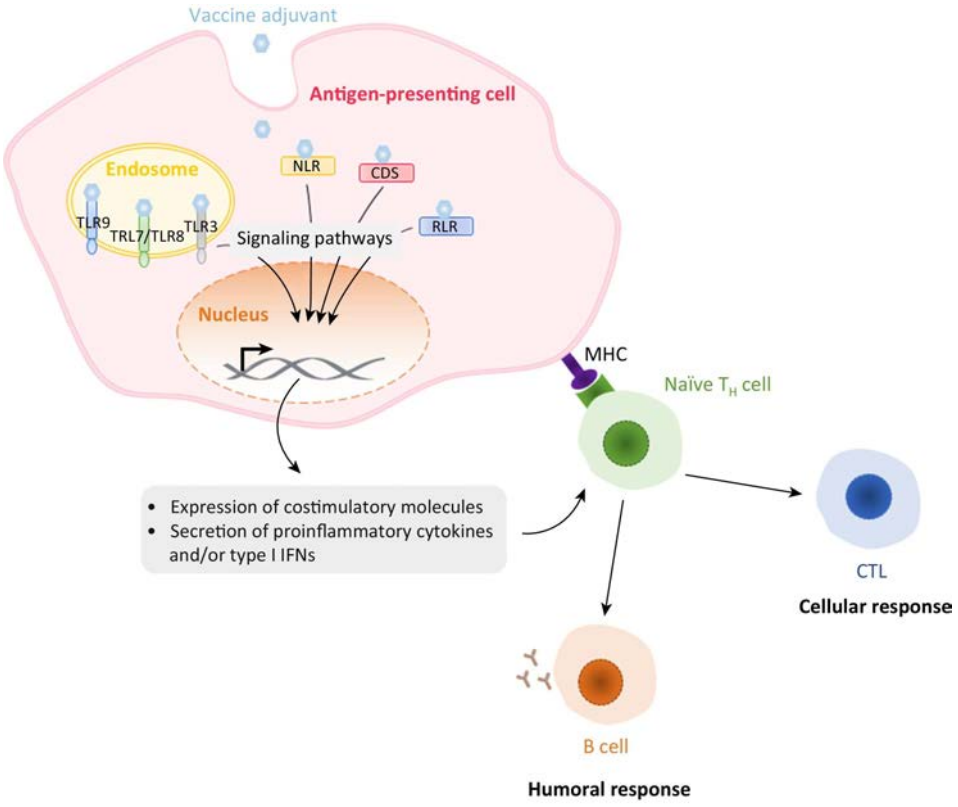

FIGURE 7: Mechanisms of Action of Adjuvants Targeting Intracellular Receptors, reprinted with permission from (99). The adjuvant is a pattern-recognition receptor (PRR) ligand, which activates antigen-presenting cells to express costimulatory molecules, proinflammatory cytokines &/or type I interferons (IFNs). This induces recruitment of immune cells & evokes a CD8+ or CD4+ immune response or both. Abbreviations: CDS, cytosolic DNA sensor; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; NLR, NOD-like receptor; RLR, RIG-like receptor, TH cell, helper T cell; TLR, Toll-like receptor

The majority of commercial vaccines available in aquaculture against viral diseases continue to be injectable, inactivated IWV vaccines formulated in oil adjuvants (**Table 4**). Historically, these oil-containing emulsions were associated to adverse inflammatory reactions at the injection-site (51), although they do induce strong long term protection. Current oil adjuvants such as Montanide emulsions are much improved (100) but alternatives are being sought. One approach is to use PAMPS as agonists of Toll-like receptors or other PRRs to induce inflammatory cytokine release and other innate immune responses. For example, unmethylated CpG dinucleotides (CpG motifs) were introduced into a DNA vaccine encoding the VHSV-G protein which was injected i.m. into fingerling trout. Significant upregulation of genes encoding type 1 IFN was observed in muscle and spleen and MHC I in spleen. Moreover, the serum neutralizing capacity against VHSV increased as more copies of the motif were introduced into the vaccine (101). Chemokines and cytokines are also being tested (100). Chang, 2015, greatly enhanced the performance of a DNA vaccine encoding hemagglutinin-esterase of ISAV by co-injecting i.m. several plasmids with Atlantic salmon type 1 IFN (a, b and c). IgM antibodies against ISAV were increased and post challenge the RPS was > 90% with the vaccine plus molecular adjuvant, compared to RPS 60% with the vaccine alone. Real-time PCR studies also showed an increased influx of B-cells and cytotoxic T-cells at the muscle injection site when the IFNs adjuvant was included (67). **Figure 8** lists positive outcomes of using a good adjuvant.

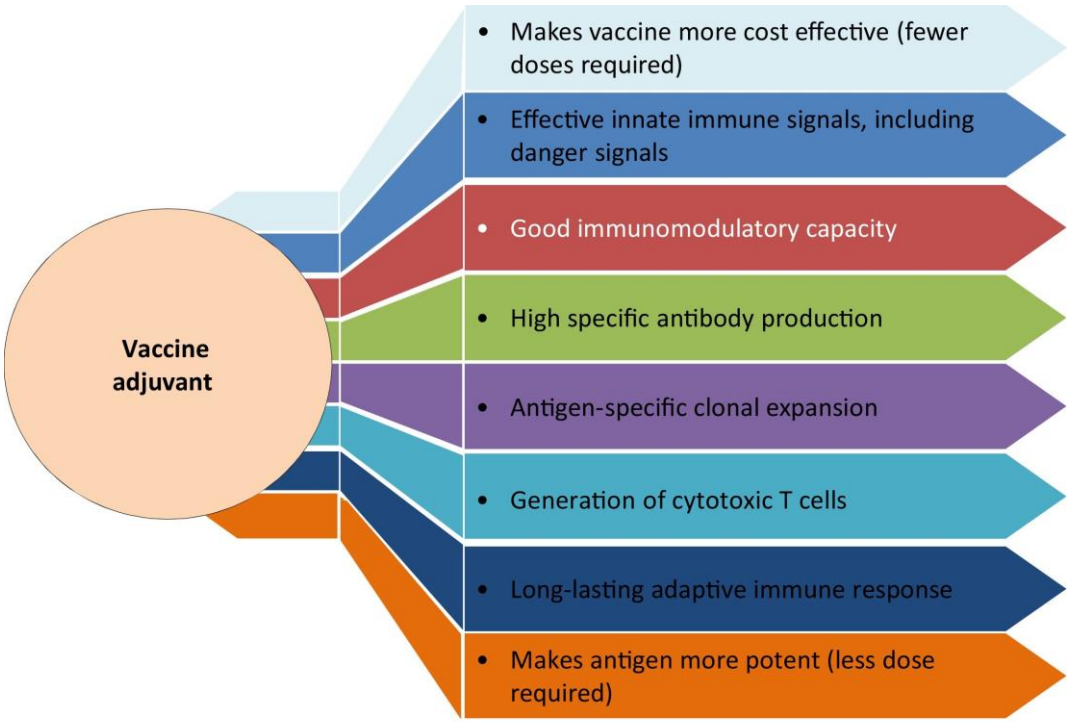

FIGURE 8: Characteristic properties of vaccine adjuvants. Reprinted with permission from (102).

For mucosal vaccines, β-glucans found in the cell wall of the yeasts *Candida albicans*, *Saccharomyces cerevisiae* and in certain plants are considered promising adjuvant candidates, which should be explored for fish oral vaccines (58). They can be easily mixed into the feed and have shown immune enhancing effects, but the mechanism of action is unclear. Their use as an immuno-stimulant for fish is reviewed in (103). Lipopolysaccharide (LPS) and enterotoxins are also candidates as mucosal adjuvants as small amounts trigger strong mucosal responses. Perez, 2013, has developed a microparticle adjuvant for aquaculture, containing LPS from meningococcal bacteria. Fed to catfish (*Clarias gariepinus*) in conjunction with formalin-inactivated *Aeromonas hydrophilia* vaccine, levels of IgM increased significantly (104). It will be interesting to see results for such adjuvants used with oral vaccines against viruses.

5. Three viruses targeted in this thesis: IPNV, VHSV and VNNV

To select the viruses we would target in this project, a literature search was performed, taking into account the set of inclusion criteria presented in **Table 5.** We decided to choose 3 viruses as a starting point in order to have several options available. If there were experimental difficulties with a particular construct or logistical problems in organizing *in vivo* testing in farmed fish species with collaborators, there would be some flexibility.

TABLE 5: Inclusion criteria for fish viruses in this project

	Inclusion Criteria
1	The virus causes significant losses to the European aquaculture industry.
2	There is no treatment, or the current treatment is labor intensive (e.g. injection), costly or raises ethical issues (e.g. DNA vaccines)
3	Immunogenic proteins of the virus have been identified and their sequences are publically available via Genebank NCBI, or Uniprot.
4	Affected farmed species should include both fresh water and marine finfish
5	Collaborators are available to do *in vivo* tests with farmed fish species.

The three viruses chosen were Infectious pancreatic necrosis virus (IPNV), Viral hemorrhagic septicaemia virus (VHSV) and viral nervous necrosis virus (VNNV or NNV) whose basic characteristics are given in **Table 1** of this chapter. **Figure 9** shows the geographical distribution and species affected by the 5 main viruses causing serious diseases in farmed finfish in Europe (11), including the 3 chosen for the project in the black, orange and blue circles.

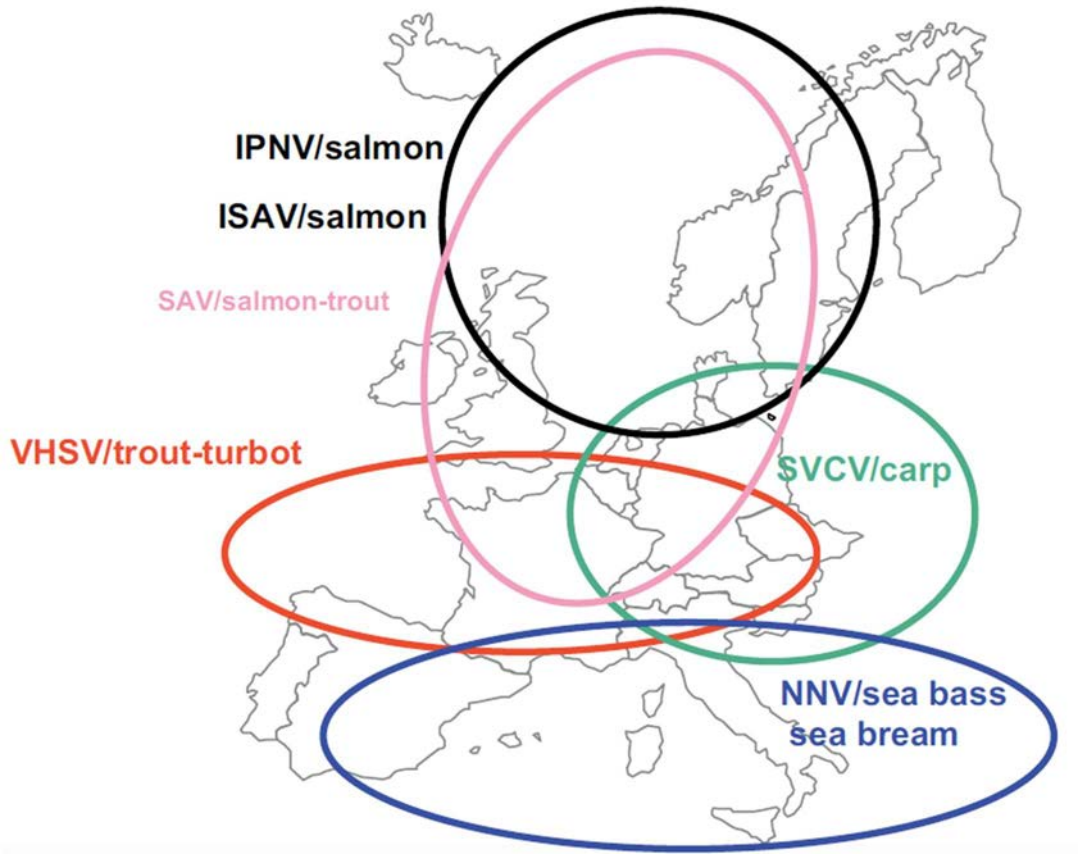

FIGURE 9: Species affected, and approximate geographical distribution of the main viral diseases in European farmed finfish caused by IPNV, ISAV, SAV, VHSV, SVCV and NNV; reprinted with permission from (11).

In relation to the inclusion criteria, **Table 6** provides information about the 3 viruses chosen and their immunogenic proteins.

TABLE 6: Three viruses chosen for this project

Virus	Main species affected	Impact	Vaccine available in Europe	Immunogenic protein, source	Collaborating centres
IPNV	Atlantic salmon, trout	Medium, resistant salmon strains have been bred	Yes. Inactivated virus vaccine, i.p. injected. Oral vaccines exist but not licensed in Europe.	Viral capsid protein 2, VP2 Uniprot KB Q703G9	INIA, Madrid
VHSV	Trout, turbot	High, OIE listed	No Experimental vaccines only	Glycoprotein G Uniprot KB P27662	UMH, Elche
VNNV reassortant strain RGNNV/SJNNV	marine fish sea bream, Senegalese sole, turbot	high, emerging strain	Not for emerging strain. Inactivated virus vaccine, i.p. injected for RGNNV strain for sea bass in Mediterranean	C coat protein NCBI GenBank NC_024493.1	IFAPA, Cadiz

In the case of IPNV, a quantitative trait locus (QTL) has been determined that accounts for most of the genetic variation in resistance to the virus. It was mapped to the epithelial caderin (*cdh1*) gene region, whose protein Cdh1binds IPNV virions in co-immunoprecipitation assays and is thought to be involved in virus internalization mechanisms (105). Using this genetic marker for resistance to IPN, selective breeding has been underway in Norway, and a great reduction in IPN outbreaks has been seen. Nevertheless, the Norwegian Veterinary Institute is still reporting significant problems in some broodstock farms and hatcheries (106). Apart from **Table 1 and 6** of this chapter, more information will be given about the viruses and their immunogenic proteins in the next chapters. In **Table 6** the institutions listed as collaborators are: INIA - Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria; UMH - Universidad Miguel Hernandez de Elche and IFAPA – Instituto de Investigación y Formación Agraria y Pesquera. Finally experiments planned at INIA were cancelled due to an outbreak of white spot disease (probably due to the parasite *Ichyophthirius multifilis*) in the trout installation. All fish had to be culled.

6. Bacterial inclusion bodies (IBs) as a tuneable therapeutic tool

Bacterial inclusion bodies (IBs) are protein nanoparticles formed in recombinant cell factories when bacteria are induced to produce large amounts of protein rapidly. In *Esherichia coli* the production yield of a heterologous protein can be greater than 50% of the total cellular protein (107). This is a very stressful scenario for the bacteria, and a very crowded cytosol. The folding apparatus of the bacterial cell is unable to cope under these conditions and protein aggregates known as IBs build up. IBs are routinely seen in recombinant protein production. They are typically ~50-800 nm in size and are a heterogeneous tangle of protein in different stages of folding/ aggregation. The culture conditions play a critical role in their final composition, especially temperature (108, 109) Approximately 20% is an amyloid-like scaffold of β sheets which is resistant to digestion by proteinase K (110) and provides stability. The rest is misfolded, semi-folded and native-like folded protein (**Figure 9**). IBs are porous and the "trapped" protein is released slowly over time, retaining functional activity. This has been demonstrated with rescue studies in which damaged cells were treated with IBs of the protein they required, and recovery was observed. For example, mammalian cells undergoing cisplatin-induced apoptosis were rescued from dying by addition of IBs made of Hsp70, an anti-apoptopic protein (111). IBs are therefore an active biomaterial which can be made of the protein of choice and act as a protein depot. They are non-toxic and penetrate cells spontaneously through macropinocytosis (112) and can function as protein releasing agents in the cell (108). These properties are being explored for therapeutic use, for example in anticancer research. In a recent proof of concept study, IBs formed by a potent CXCR4 antagonist were injected into CXCR4+ colorectal tumors in mice. This resulted in a marked increase in apoptosis within the tumor tissue and an inhibition of cell proliferation, not observed using non-functional IB controls (113). Another therapeutic area testing IBs is wound healing, using the lipogenase AmbLOXe from the Mexican axolotl. This amphibian (*Ambystoma mexicanum*) has an impressive regeneration and healing capacity and the gene AmbLOXe is upregulated during limb regeneration. IBs of AmbLOXe were produced in *E.coli* and were used in *in vitro* would healing assays with HaCaT keratinocytes. Faster migration rate and wound closure was observed in cultures with the functional IBs in the medium or coated

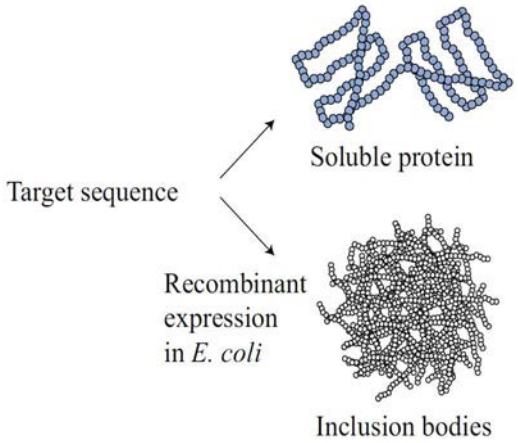

FIGURE 9: Representation of inclusion bodies. Depending on the protein and culturing conditions, recombinant proteins will form IBs. Adapted from (109), BMC open access.

on cell surfaces (114). Given these findings, the lack of intrinsic toxicity and ease of production, we were keen to investigate IBs as protein releasing agents for fish.

6.1 IBs as immunostimulants for fish

As part of the simple production and purification process in *E.coli*, IBs retain some bacterial impurities. Typically, lipoploysaccharide (LPS), petidoglycan (PGN) from membrane and cell wall fragment, as well as DNA and RNA will be present in variable quantities, though our methods ensure there is not viable bacterial cell contamination (115). These bacterial remnants are well-known immunostimulants for fish (116, 117). Torrealba 2016, showed that IBs made of an immune irrelevant protein, iRFP (infra-red fluorescent protein), injected i.p. to zebrafish, provided a degree of short term protection against infection with *Pseudomonas aeruginosa,* reaching a maximum RPS of 48%. When the IB was made with the cytokine TNFα from rainbow trout, the RPS reached 100% (118). This indicates that a cytokine IB can provide *in vivo* protection against a pathogen. TNFα IB had a specific protective effect, but also the IB *per se* contributed to protection, presumably through immunostimulation by the bacterial impurities it contains. Further, in the same publication, the TNFα IB was subject to stability tests over a range of temperatures and pH, covering the pH range found in the fish gastro-intestinal system. The nanoparticle retained the ability to stimulate gene expression of innate immune gene markers and after lyophilization, it still provided *in vivo* protection against *P. aeruginosa* infection in zebrafish. The IB format thus showed promise for use in fish immunology.

7. Our new vaccine strategy: Fish viral antigens nanostructured as IBs

In this project we take the IB strategy from immunostimulant to vaccine. We design and produce protein nanoparticles of fish viral antigens structured as IBs and test their potential as a fish vaccine. The recombinant protein is produced in a basic, scalable system and purification is simple, thus manufacturing is cheap. The format is a stable protein that could be mixed in the feed and is innocuous for fish. These nanoparticles have the unique property of being the delivery vehicle and antigen as one, as well as carrying immunostimulants. In the following chapters we investigate this new approach.

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OBJECTIVES

The aquaculture industry is an important sector worldwide in food production and food security. Viral diseases are one of the main challenges the industry faces, yet the tools available to combat them are limited and costly. Therefore:

*The **overall aim** of this work is to develop a new vaccine strategy based on recombinant protein nanoparticles with view to providing a platform for effective, practical vaccine production for farmed fish.*

This project targets three viruses relevant for European finfish farming. The **main objectives** are:

1. Design and produce bacterial inclusion bodies in *Escherichia coli* made of antigenic proteins of the following fish viruses: infectious pancreatic necrosis virus (IPNV), viral haemorrhagic septicaemia virus (VHSV) and viral nervous necrosis virus (VNNV). In this way, stable nanostructured viral protein antigens would be obtained.

2. Test these recombinant protein nanoparticles *in vitro* for fundamental information regarding uptake by fish cells, cytotoxicity and the capacity to stimulate gene markers of the anti-viral innate immune response.

3. Test uptake of the nanoparticles *in vivo* via the intestine in zebrafish as an experimental model for oral delivery and monitor macro-toxicity.

4. Administer the nanoparticles *in vivo* as an experimental vaccine in farmed fish species models and test for indicators and surrogates of protection. These include production of specific antibodies against the target virus or viral protein, upregulation of genes related to the adaptive immune response, survival post infection challenge and reduction in viral load post infection.

5. Explore the *in vivo* protection achieved by administering the nanoparticles by different routes in farmed fish species models. Compare the surrogates of protection evoked by injection and oral delivery.

CHAPTER 1

NANOPARTICLE PRODUCTION AND PROOF OF CONCEPT STUDIES

Chapter based on publication:

Protein nanoparticles made of recombinant viral antigens: a promising biomaterial for oral delivery of fish prophylactics

Thwaite R, Ji J, Torrealba D, Coll J, Sabes M, Villaverde A, Roher N (2018).
Frontiers in immunology 9:1652. doi:10.3389/fimmu.2018.01652.

1. Abstract

In the search for an eminently practical strategy to develop vaccines for farmed fish, we have devised recombinant viral antigens presented as “nanopellets”. These are inclusion bodies of fish viral antigenic proteins produced in *Escherichia. coli*. Soluble recombinant proteins are too labile to endure the *in vivo* environment and maintain full functionality, and therefore require encapsulation strategies. Yet when they are produced as nanostructures, they can withstand the wide range of gastrointestinal pH found in fish, high temperatures and lyophilization. Moreover, these nanomaterials are biologically active, non-toxic to fish, cost effective regarding production and suitable for oral administration. Here we present three versions of nanopellets formed by antigenic proteins from relevant viruses affecting farmed fish: the VNNV coat protein, IPNV viral protein 2 and a VHSV G glycoprotein fragment. We demonstrate that the nanoparticles are taken up *in vitro* by zebrafish ZFL cells and *in vivo* by intubating zebrafish as a proof of concept for oral delivery. Encouragingly, analysis of gene expression shows these nanopellets evoke an anti-viral innate immune response in ZFL cells and in rainbow trout head kidney macrophages. They are therefore a promising platform for immunostimulants and can be candidates for vaccines should protection be demonstrated.

2. Introduction

Viral diseases are a major concern in the aquaculture industry. Vaccine strategies need to optimize efficacy, while taking into account production and administration costs, environmental risks and compliance with legislation. The traditional approach is based on the use of inactivated or attenuated viral vaccines, which are commercially available for certain viral diseases that cause high mortality (1). Such vaccines induce a strong immune response when combined with oil adjuvants (2). However, not all fish viruses are readily culturable in cells, for example lymphocystis disease virus (3), and the process is expensive, with administration via injection, or immersion for juveniles. Another consideration is the risk of possible reversion to virulence and environmental spread. New strategies are thus being sought. Among them, recombinant DNA vaccines have achieved promising results against certain viruses (4, 5) but raise safety issues regarding genetically modified organisms (6). In fact, only one DNA vaccine, Clynav® (Elanco) against salmonid alphavirus subtype 3, has been recently licensed in Europe. Like other DNA vaccines, it is administered by labor intensive intramuscular injection. Injection is costly and difficult to perform on juveniles, as well as causing stress and injury to fish. An alternative vaccine approach is the use of recombinant protein viral antigens. These subunit vaccines can be produced in bulk, but have been variable in efficacy (1). One promising format, virus-like particles (VLPs), uses self-assembling viral capsid proteins produced in yeast, bacteria or cell culture, drawing on

advances in human and animal vaccinology (7, 8). The main advantage of subunit vaccines is they are safe. There is no risk of DNA integration into the host, reversion or invasion. The main drawback is the stability and half-life of recombinant proteins *in vivo*. Oral delivery would be the most practical, least stressful delivery method, however immune-relevant epitopes need to be protected against gastrointestinal pH, which is particularly low in carnivorous fish (see fig 1 in (9)), as well as digestive enzymes within the tract. Thus different encapsulation techniques such as alginate and chitosan are being tested, aiming to protect the recombinant protein antigens from rapid degradation when inside the animal (10).

Here we present a novel approach to finfish prophylactic design. To enhance the stability of antigenic proteins while maintaining functionality, we have nanostructured viral protein antigens as bacterial inclusion bodies (IBs). IBs are highly stable, tuneable, nanoscale protein particles which can penetrate cells, while retaining significant biological activity, as demonstrated by rescue studies (11). They can be designed to bear the antigenic protein/ epitopes of interest and provide a slow release of functional protein over time (12). The attractiveness of IBs as a fish prophylactic is manifold. Their stability at gastrointestinal pH (13) would allow administrating the antigen orally through the feed, avoiding the necessity for vaccine encapsulation and the cost and stress to fish associated with injection. Production in *E. coli* is achieved in bulk with a simple enzymatic and mechanical purification procedure which minimizes costs (14). This straightforward process implies that the IBs carry over fragments of bacterial lipopolysaccharide, peptidoglycans and nucleic acids as impurities, but which are known adjuvants and immunomodulators of fish (15). The IB vehicle, a carrier and viral antigen as one biomaterial, should elicit both an innate and adaptive immune response against the target virus in fish. Finally, IBs' stability under lyophilizing conditions and over a range of temperatures (13) indicates their potential as a practical farm product with a lasting shelf life, avoiding the cold-chain.

We have already demonstrated the potential of IBs as an immunostimulant for fish, by nano-structuring recombinant cytokines TNF-α and CCL4 and testing them in a bacterial infection model in zebrafish (13). In addition, uptake of the TNF-α IB by intestinal cells was demonstrated *in vivo* in rainbow trout via oral intubation (13). This paved the way for work focusing on producing viral antigens as IBs, to explore this approach for immune-stimulus, and ultimately as a viral vaccination strategy.

This paper is a proof of concept study concerning the production, uptake *in vitro* and *in vivo* and innate immunogenic potential of fish viral antigens configured as recombinant IBs. Given our final aim is their use in fish food, we have coined the term "nanopellets" (NPs) to refer to these novel nanostructured antigens. We chose three target proteins of known antigenicity from significant viruses affecting farmed finfish, reviewed in (6). They are the viral capsid protein 2

(VP2) from infectious pancreatic necrosis virus (IPNV) an *Aquabirnavirus* causing high mortality in young salmonids, the glycoprotein G of viral haemorrhagic septicaemia virus (VHSV), a *Novirhabdovirus* which is a current OIE listed fish viral disease (http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2019/) primarily affecting farmed trout and turbot, and the C coat protein of viral nervous necrosis virus (VNNV), a *Betanodavirus* affecting sea bass, sea bream, flounder and sole, among many other fish (16). We show the NPs can be produced by cost-effective, reproducible methods and can be taken up in ZFL (zebrafish liver cell line) and *in vivo* by zebrafish (*Danio rerio*) when orally administrated. Moreover, we show the viral antigen NPs can evoke an immune response *in vitro,* upregulating gene markers of the innate viral immune response in ZFL and rainbow trout (*Oncorhynchus mykiss*) head kidney macrophage cell cultures (RT-HKM).

3. Materials and Methods

3.1 Design, Production and Characterization of Nanostructured viral antigenic proteins

3.1.1 Viral strains and plasmids

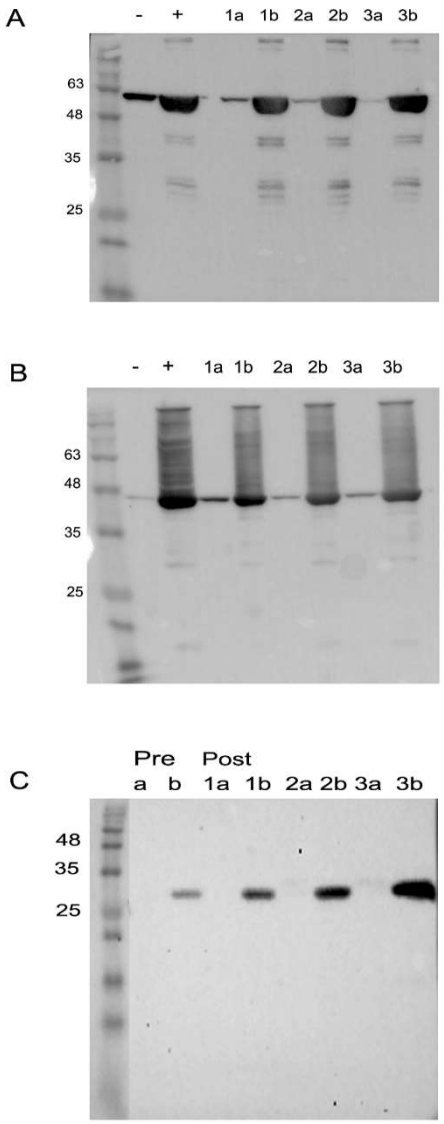
For the three target viruses, sequences for the antigenic proteins of interest were: VNNV coat protein gene from the Iberian betanodavirus isolate (strain SpSs-IAusc160.03), NCBI GenBank, accession no: NC_024493.1 which is a reassortant RGNNV/SJNNV strain (17); IPNV capsid protein 2 from the infectious pancreatic necrosis virus (strain Sp 31-75), UniprotKB Q703G9 Chain (PRO_0000227873) position 1-442; VHSV glycoprotein G from the viral hemorrhagic septicaemia virus (strain 07-71), Uniprot KB P27662. Clones were designed using the ORF and pET22b in a strategy removing the periplasmic location signal and including a C terminal polyHistag. Clones were codon optimized for expression in *E. coli*, synthesized by GeneArt (Thermo Fisher Scientific) and subcloned into pET22b. Recombinant plasmids were transformed into *E. coli* BL21(DE3) (Novagen). Upon protein production (section 3.1.2) the VHVS-G protein showed hallmarks of being toxic for *E. coli,* with slow host growth and scant protein yield post production (data not shown). This clone was substituted by VHSV-G-frg16 cloned into pRSETa, which covers the C-terminal half (amino acid residues 252-450) of the VHSV (07-71) G protein sequence (NCBI Genbank X59148) to the 3'end, with the Cys residues mutated to Ser to facilitate expression in *E. coli*. The sequence includes a putative integrin receptor RGD binding site and 2 regions which induce *Mx* gene expression (18, 19). Further, frg16 is able to bind specific anti-VHSV rainbow trout antibodies in fish surviving VHSV infection (20). Apart from the viral antigen constructs, a construct with the red fluorescent protein (RFP), iRFPHis cloned in pET22b (Genscript), was also transformed into *E. coli* BL21(DE3) to be used as a non-immune-relevant

control protein. Images of this protein as an IB, which we call iRFP^{NP}, and its emission spectra can be found in the Supplementary material Annex I.

3.1.2 Production of NPs, purification, quantification and fluorescent labelling

Production of nanostructured viral and control proteins from the clones transformed into *E. coli* followed methods described in (13). Successful production of insoluble protein (IBs) in the predicted size range was confirmed by Western blot **Figure 1**, before large-scale production.

FIGURE 1: Western blots to check NP protein production in soluble & insoluble fractions



Cultures were induced with 1 mM IPTG. Samples were taken pre-induction & every hour post induction for 3 h. Cells were collected by centrifugation, resuspended in PBS plus protease inhibitor (cOmplete Tablets, Roche) and sonicated (total protein production). To obtain soluble & insoluble fraction, equivalent amounts of cells (volume of cell culture adjusted to the same OD for every time point in A and B) were centrifuged, resuspended in PBS & sonicated. Sonicated cells were centrifuged 14,000 x *g* at 4ºC for 15 min. Supernatant was retrieved as the soluble fraction; the pellet, (insoluble fraction) was resuspended in the same volume of PBS. Equal aliquots were run on a western and NPs detected using an anti-His-tag antibody.

A and B: Production of IPNV-VP2^{NP} and VNNV-C^{NP} respectively.

Total protein production pre-induction (-) & 3 h post IPTG induction (+) for the same volume of culture sample. Soluble (a) & insoluble (b) fractions at 1, 2 & 3 h post IPTG induction for the equivalent amount of cells at all time points.

C: Production of VHSV-G-frg16^{NP}.

Soluble (a) & insoluble (b) fractions, pre-IPTG induction & 1, 2, 3 h post IPTG induction for the same volume of culture at all time points.

Note: The amount of protein increases sharply with IPTG induction & protein is almost entirely in the insoluble fraction (inclusion bodies). Predicted molecular weight of each protein using Expasy Protparam is 49.6, 38.2 & 30.4 kDa, for A, B, C.

Nanoparticle production was as follows: *E. coli* was cultured in LB with ampicillin (Sigma) at 100 µg/mL and recombinant protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Panreac) when $OD_{550\ nm}$ reached 0.5-0.8. After a further 3 h growth at 37ºC, lysozyme (Sigma) (1 µg/mL), protease inhibitor (cOmplete Tablets, Roche) and phenyl-methanesulfonyl fluoride (Roche) (0.4mM) and were added and incubation continued at 37ºC for 2 h at 250 rpm. Cells were then frozen at -80ºC overnight (O/N). After thawing, Triton X-100 was added to 0.2% v/v and the mix was stirred for 1 h at room temperature (RT). IBs were harvested by centrifugation at 15000 x *g*, 15 min, resuspended in lysis buffer (50 mM Tris HCl, 100mM NaCl, 1 mM EDTA) at 1/10 the original culture volume and treated with DNAse (Roche) (0.6 µg/mL) at 37ºC with shaking. Then several freeze thaw cycles were performed to rupture any remaining viable bacteria. Finally, the nanostructured proteins were subject to sterility tests without antibiotic on LB-agar overnight and in DMEM culture medium (Gibco) at 37ºC for 3 days. Pellets of purified NPs were obtained by centrifugation at 15 000 x *g* for 30 min, 4ºC. They were named IPNV-VP2^{NP}, VHSV-G-frg16^{NP} and VNNV-C^{NP} and were stored at -80ºC until use. Protein was quantified by western blot using an anti-His-tag antibody (Genscript A00186-100) and the protein concentration was calculated from a standard curve using recombinant protein and Quantity One software (Biorad). Quantification was further tuned via spectrometry by comparing 100 µg/mL dilutions of the different NPs at 320 nm and using the correction factor determined to adjust the quantification accordingly. For experiments to visualize the nanoparticles by flow cytometry or confocal microscopy, NPs were conjugated with Atto-488 NHS ester (Sigma-Aldrich) according to the manufacturer's instructions and (21). Labelling efficiency was determined on a fluorometer (Jasco FP8200). Equal volumes of nanoparticles at 100 µg/mL were treated with 6 M guanidinium chloride (Sigma-Aldrich) to denature overnight (O/N) at room temperature (RT) in the dark and the fluorescence intensity was read the next morning **Figure 2**.

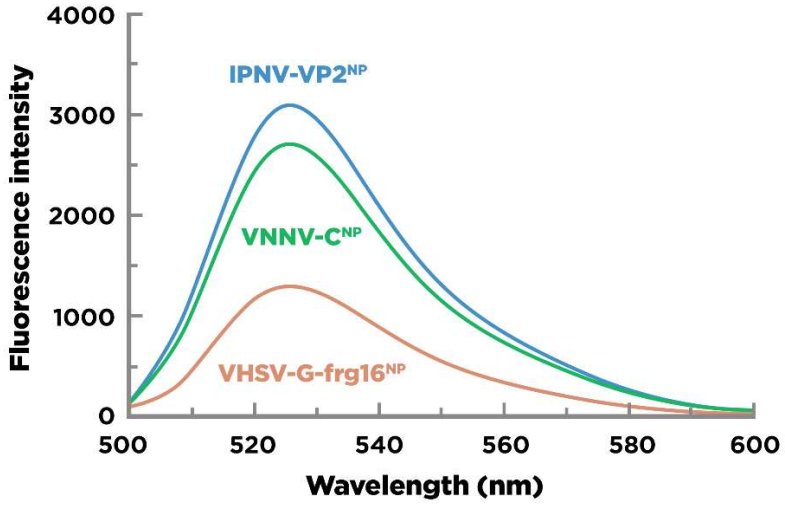


FIGURE 2: Efficiency of fluorescent labeling of NPs with Atto-488 NHS ester. NPs at 100 µg/mL were treated with 6 M guanidinium chloride & fluorescence intensity read on Jasco FP8200 fluorometer.

3.1.3 Characterization of Viral Recombinant Protein NPs

We used Field Emission Scanning Electron Microscopy (FESEM, Zeiss Merlin) to determine the external morphology and physical dimensions of the NPs. Samples were prepared by resuspending NPs at 100 µg/mL in distilled water, pipetting 20 µL onto silicon chips and airdrying O/N. Images were analyzed using Fiji open source image processing package (22), measuring the dimensions of a minimum of 120 particles for each construct. Size distribution histograms were generated using Past3 software (v3.18, University of Oslo).

3.2 In vitro assays

3.2.1 Cell cultures

Zebrafish ZFL cells (CRL-2643, ATCC) were cultured according to (23) at 28 ºC and 5 % CO_2 in DMEM+GlutaMAX (Gibco), 10 % heat inactivated fetal bovine serum (FBS) (Gibco), 0.01 mg/mL insulin (Sigma-Aldrich), 50 ng/mL epidermal growth factor (EGF) (Sigma-Aldrich), 2% (v/v) antibiotic/antimycotic (Gibco) and 0.5% (v/v) trout serum which had been filtered (0.20 µm filter Corning) and heat inactivated for 30 min at 45ºC, before storing at -20ºC. Rainbow trout head kidney macrophages (RT-HKM) were isolated from *O. mykiss* (109 ± 18 g body weight) following previously described procedures (24). Primary adherent cultures were established in DMEM+GlutaMAX, 10% heat inactivated FBS and 100 µg/mL Primocin (Invitrogen) at 16ºC and 5% CO_2. Experiments for NP uptake and gene expression were performed on day 5 when the macrophages were fully differentiated.

3.2.2 Uptake of nanostructured viral antigens by ZFL

To test cellular uptake, fluorescently labelled NPs were added to ZFL cultures at 70% confluence after 2-3 h incubation in minimal media (0 % FBS) at the doses and times indicated below. For dose response assays, VNNV-C^{NP} and IPNV-$VP2^{NP}$ were added at 5, 10 and 20 µg/mL; and VHSV-G-$frg16^{NP}$ at 1, 5, 10 and 20 µg/mL. Cultures were then incubated O/N (12-14 h). In time course experiments NPs were added at 10 µg/mL for VNNV-C^{NP} and IPNV-$VP2^{NP}$; and at 5 µg/mL for VHSV-G-$frg16^{NP}$ and cultures were simultaneously incubated for 6-48 h before harvesting. Both dose response and time course experiments were performed in duplicate. Post treatment, cells were washed in phosphate buffered saline (PBS) and incubated at 28ºC with 1 mg/mL Trypsin (Gibco) for 15 min. This strong trypsinization step aimed to remove NPs attached to the cell surface (25). Then, two volumes of complete medium were added, and cells were retrieved by centrifugation at 300 x *g* for 5 min. Pellets were resuspended in PBS for flow

cytometry (FACSCalibur BD) and 10,000 events were counted. Data was analyzed using Flowing Software 2.5.1 (University of Turku, Finland) and plotted with Prism 6.01 (GraphPad). A one-way ANOVA was performed with Dunnett's multiple comparisons test, comparing treatment and control means. To confirm the fluorescent NPs were inside the cells, we performed confocal microscopy (Zeiss LSM 700). ZFL cells were seeded on Nunclon Δ Surface individual well plates (Nunc). The next day cells at approx. 60% confluence were placed in minimal media. NPs were added 2-3 h later as follows: VNNV-C^{NP} and IPNV-$VP2^{NP}$ at 20 µg/mL and VHSV-G-$frg16^{NP}$ at 10 µg/mL. Cells were incubated for 14 h at 28ºC. Media was replaced with minimal media in which the cells were stained with DAPI (nuclei) and Cell mask Deep Red (membrane) (Life technologies). Images were analyzed using Imaris software v8.2.1 (Bitplane).

3.2.3 NP cytotoxicity studies in ZFL

Cytotoxic and cytostatic effects of NPs on ZFL were checked using an MTT (3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After 2.5 h on minimal media, cultures were stimulated with NPs at 10, 20 and 50 µg/mL and incubated for 14 h at 28 ºC. Cells were washed in PBS and MTT substrate (Sigma-Aldrich) was added to 10 % total volume. Controls were: cells with no NPs; cells with no NPs but treated with 1 % Triton before adding MTT; and cells with no NPs and no MTT. Cells were further incubated at 28 ºC for 6 h. The solution was removed, cells were solubilized in DMSO and the lysate read on Victor 3 (PerkinElmer) at 550 nm. The experiment was repeated twice. Data was normalized using Prism 6.01 (Graph Pad) such that the control readings were set at 100 % and the Triton treatment readings were 0 % viability, which was equivalent to cells without MTT. A one-way ANOVA was performed with Dunnett's multiple comparisons test, comparing treatment and control means.

3.2.4 Gene expression analysis in ZFL and RT-HKM treated with NPs

ZFL cells at 60 % confluence were cultured in minimal media (0-0.5 % FBS) for 2-3 h and then stimulated for 14 h with NPs at the following concentrations in triplicate: VNNV-C^{NP} and IPNV-$VP2^{NP}$ at 10 µg/mL, VHSV-G-$frg16^{NP}$ at 5 µg/mL. Controls were poly (I:C) 25 µg/mL (Sigma-Aldrich) as a viral dsRNA mimic and red fluorescent protein nanopellet $iRFP^{NP}$ at 10 µg/mL as an immunogenically irrelevant protein, as well as control cells with no stimulus. Total RNA was extracted using TriReagent (Sigma-Aldrich) following manufacturer's instructions. RNA was quantified on nanodrop ND-1000 (Thermoscientific) and integrity checked on the Agilent 2100

Bioanalyzer using the RNA 6000 Nano Lab-Chip kit (Agilent Technologies). The experiment was repeated and four complete sets of high quality RNA from two independent experiments were selected for cDNA synthesis using 1 µg of total RNA and iScript cDNA synthesis kit (BioRad). Quantitative real-time PCR (qPCR) was performed at 60 ºC annealing temperature using iTaq Universal SYBR Green Supermix (BioRad) with 250 nM of primers and 2.5 µL of cDNA previously diluted to 1:25 for the target and 1:500 for the reference gene, elongation factor 1 alpha (*ef1-α*) (26). Primers were designed for 6 zebrafish gene markers of the innate immune response to viral infection (*mx, viperin, gig 2, irf7, stat1b and ccl4*) using NCBI Primer BLAST, and revised using Oligoanalyzer 3.1 (Integrated DNA Technologies). The primer sequences and accession numbers are listed in **Table 1**. All the samples (N=4 per treatment) were run in triplicate and data was analyzed for individual replicates using the Livak method (27). Statistical analysis used a one-way unpaired t-test to compare each gene's mean fold change in expression with control using Welch's correction for unequal variances (Prism 6.01, GraphPad).

A further gene expression experiment was done in RT-HKM primary cultures using the 2 NPs made with antigenic proteins from virus affecting salmonids, IPNV and VHSV. Macrophage cultures were prepared as described in section 3.2.1. On day 5, cultures from 3 trout at approximately 70% confluence were placed in serum free media for 2 h at 16ºC. Cultures were stimulated for 15 h as follows: IPNV-VP2^{NP} and VHSV-G-frg 16^{NP} at 10 µg/mL; and controls: poly (I:C) at 10 µg/mL and iRFP^{NP} at 10 µg/mL, as well as cells with no stimulus. The experiment was repeated twice. Total RNA was extracted and quantified as described above for ZFL. From the 2 independent experiments, 4 sets of high quality RNA were selected for cDNA synthesis and qPCR as above. The trout primer sequences were obtained from published papers or were designed with NCBI primer BLAST, selecting genes reported to be upregulated in VHSV infection of *O. mykiss* (28). Reference gene used was *ef1-α* (29) with cDNA diluted to 1:500. The dilution factor for the other genes tested was 1:50 (*vip1, mx, ccl4*) or 1:25 (*ifit5, mda5*). Primer sequences and accession numbers are listed in **Table 1**. Data analysis was performed as above.

3.3 *In vivo* assays

3.3.1 Animals

Adult wild-type zebrafish (*D. rerio*) and rainbow trout (*O. mykiss*) fish were maintained at 27 ±1ºC and 17± 1ºC respectively in a 12 h light/dark cycle, fed twice daily with a commercial diet at 2 % ratio. All experimental procedures were approved by the Human and Animal Experimentation Ethics Committee of the Universitat Autònoma de Barcelona (Reference 1533) and were done in strict accordance with the recommendations of the European Directive (2010/63/EU) on the protection of animals used for scientific purposes.

Table 1: Primers used for real time PCR

Zebrafish primers			
Gene	**Sequence (5´-3´)**	**Product size** (bp)	**Accession # / reference**
ef1-α	FW_CTTCTCAGGCTGACTGTGC	133	AY422992
	RV_ACGATCAGCTGTTTCACTCCC		
mx	FW_ACATCTTGGATCGTTCAGGGGA	163	NM_182942.4
	RV_AACGCAGGTTCCTCCAACAG		
***viperin* (*vig1/ rsad2*)**	FW_CTTATAGGTCGAGCACAGGGC	165	NM_001025556.1
	RV_ACGTACTGGATTGAGAGCGGTG		
gig2	FW_AGGGTACGACACTGCCTGGT	148	NM_001245989.1
	RV_ AGGGTCACCAAAGCCACAAT		
irf7	FW_GAGCAAATACGCTTCCCGA	141	NM_200677.2
	RV_CTTGTCCTGACGAAAGCCATA		
stat1b	FW_TCCCAATGGAGATCCGACAAT	107	NM_200091.2
	RV_CAGGAGCTCATGGAAGCGAAC		
ccl4	FW_CATGACAAGCCAGCAGTGCC	126	NM_001129894.1
	RV_ACACGTTTGCTGTCAATGGCCTG		
Rainbow trout primers			
ef1-α	FW_CAAGGATATCCGTCGTGGCA	327	NM_001124339.1
	RV_ACAGCGAAACGACCAAGAGG		(29)
mx	FW_ATGCCACCCTACAGGAGATGAT	127	NM_001171901.1
	RV_TGCAGCTGGGAAGCAAACTCC		
vig1	FW_AACGCTGGGGAGAACAGTCT	181	NM_001124253.1
	RV_TCCCCTCTCGGCAATCCA		
ifit5	FW_GGGTAGCCTATTCCGCGTACTT	80	NM_001124333
	RV_CTGCTTTGACCGAGGCACTC		
mda5	FW_ TTTGTGCTGAGCATCTACGG	148	NM 001195179.1
	RV_TTAATGATGGCCTCCTCGTC		
ccl4	FW_TGTTCACCCCTCGTCTTGCT	104	NM_001124489.2
	RV_ ACATTTCTTCGGTCCGCTTG		

3.3.2 Uptake of NPs by zebrafish via oral gavage

To test *in vivo* uptake, fluorescently labelled NPs were orally intubated in zebrafish adults for the indicated times and doses, mimicking an oral vaccine administration route. Zebrafish adults (mean weight 0.9 ± 0.2 g) were acclimatized in tanks without feeding for 1.5 days prior to the experiment. Atto labelled NPs were intubated into the animals in a volume of 30 µL PBS using a gastight Hamilton syringe (Hamilton Company) with a thin silicon tube (0.30 mm inner diameter, Dow Corning) placed over the needle as a protective sheath to avoid injuring the animal. To guide oral insertion, a more rigid 10 µL filtered pipette tip end (NerbePlus) was cut and fixed over the tubing leaving the soft end exposed. Immediately prior to intubation fish were anesthetized in 120-140 mg/L MS-222 (tricaine mesylate) (Sigma-Aldrich). Preliminary small scale runs at 3, 6, 24 and 48 h at 20 µg and 50 µg/ fish indicated maximum uptake was achieved by 6 h and 20 µg/fish dose was sufficient. Then runs were performed with groups of N=8 fish for each NP at 20 µg/ fish in 30 µL PBS for 5 h. Controls were fish intubated with 30 µL PBS without NP. Post administration, fish were maintained in tanks until time of sacrifice using an overdose of MS-222. The intestine was dissected out from euthanized fish and washed in PBS. Next it was incubated in 1 mL of collagenase solution: DMEM (Gibco) with 1% v/v antibiotic/antimycotic (Gibco) and collagenase Type IV (Gibco) 1.5 mg/mL at RT on a roundabout in the dark for 1 h. The intestine was passed through a 100 µm cell strainer (Falcon, Corning), washing with PBS and cells were retrieved by centrifugation at 400 x g for 10 min at 4ºC. Cells were resuspended in PBS for flow cytometry (FACSCalibur BD) and 10,000 events were counted. Data was analyzed using Flowing Software 2.5.1 (University of Turku, Finland) and plotted with Prism 6.01 (GraphPad). A one-way unpaired t-test with Welch's correction for unequal variances, was performed to test equivalence of means between each experimental group and controls. We published the zebrafish gavage method in the Journal of Visualized Experiments (21).

3.4 Statistical analysis

Analyses were performed with Prism 6.01 software (GraphPad), and Imaris 8.2.1 (Bitplane) for the confocal images and Past3 (v3.18, University of Oslo) for data obtained from FESEM. Data is shown as means ± SD. Comparisons of means for each experimental group versus control were performed using a one-way unpaired t-test with Welch's correction for unequal variances. For the *in vitro* uptake studies and MTT tests, in which we compared a series of conditions with the same nanopellet, a one-way ANOVA was used, followed by Dunnett's multiple comparisons test for each treatment versus control; $p < 0.05$ was considered statistically significant in all analyses.

4. Results

4.1 Characterization of nanostructured viral antigenic proteins

We successfully produced the three viral proteins in *E. coli* as bacterial inclusion bodies (i.e. nanopellets, NPs) with yields post purification as follows: IPNV-VP2^{NP} 104 mg/L, VHSV-G-frg16^{NP} 120 mg/L and VNNV-C^{NP} 50 mg/L. The NPs had distinct morphologies and sizes as seen in the FESEM images, **Figure 3.** IPNV-VP2^{NP}, the largest of the NPs, is generally barrel shaped and porous; VHSV-G-frg16^{NP} is rounder and smoother, while VNNV-C^{NP} has an irregular surface with small spherical protrusions. We have observed similar morphologies in other IBs produced in *E. coli* in the same strain BL21(DE3) and in M15(pREP4) (13). The size range is shown in **Figure 3** (ii and iii) with average width and length being 607 ± 115 nm and 734 ± 195 nm for IPNV-VP2^{NP}; 488 ± 107 nm and 608 ± 121 nm for VHSV-G-frg16^{NP} respectively, and 422 ± 87 nm for VNNV-C^{NP} mean width. The morphological features of the nanostructured control protein iRFP^{NP} have already been published (14).

4.2 Uptake of viral NPs by ZFL

All three NPs were taken up by ZFL cells. In dose response experiments, uptake of VHSV-G-frg16^{NP} was found to be particularly efficient, achieving ~100% fluorescent cells at 10 µg/mL O/N (**Figure 4B** (i)). Hence an additional lower dose (1 µg/mL) for this NP was included in subsequent experimental runs. For IPNV-VP2^{NP} and VNNV-C^{NP}, uptake increased progressively with dose, reaching a maximum of ~60% and 50% fluorescent cells respectively (**Figure 4A** (i) and **4C** (i)). In all cases the mean fluorescence intensity (MFI) increased with dose, indicating susceptible cells were still able to take up more NP (**Figure 4A, B** and **C** (i) right y axis). For time course experiments a fixed dose was chosen that achieved less than the maximum uptake observed in the dose response experiments. 10 µg/mL for IPNV-VP2^{NP} and VNNV-C^{NP}, and 5 µg/mL for VHSV-G-frg16^{NP}. In the time course experiments IPNV-VP2^{NP} and VHSV-G-frg16^{NP} already reached the maximum percentage of fluorescent cells by 6 h. (Figure **4A** and **B** (ii)). For VNNV-C^{NP} uptake was slower, as the maximum percentage of fluorescent cells for the time points measured was at 24 h (Figure **4C** (ii)). In all cases, by 48 h the percentage of fluorescent cells had started to drop (Figure **4A, B** and **C** (ii)), possibly indicating the NPs had begun to be metabolized. The MFI results for the time course are consistent with this. Susceptible cells continued taking up NPs for the first 24 h, then between 24 and 48 h the MFI dropped (Figure **4A, B and C** (ii) right y axis). Note uptake by ZFL of the immune irrelevant control nanoparticle iRFP^{NP} is shown in the Supplementary material Annex I.

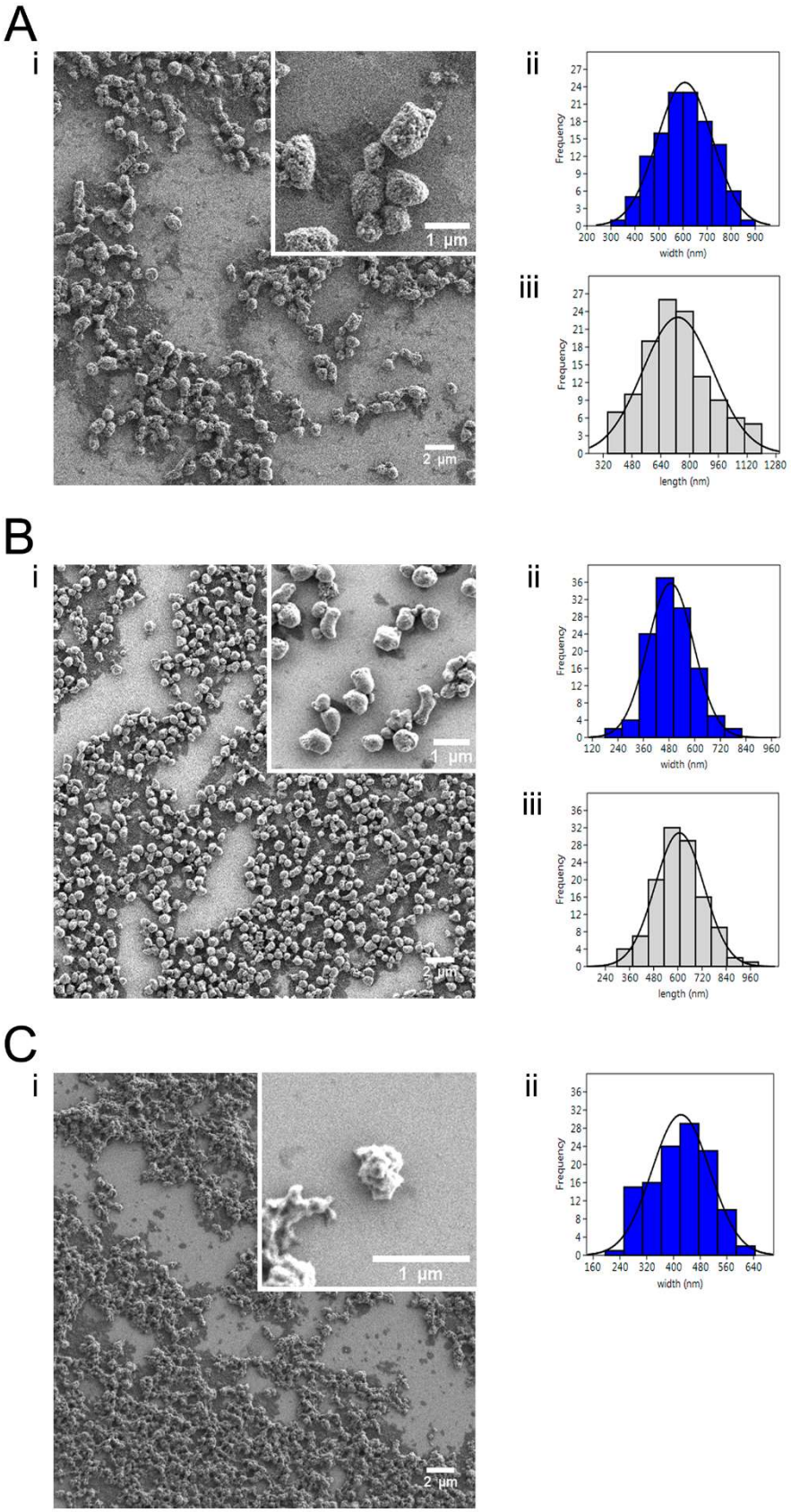


FIGURE 3: Characterization of nanostructured viral antigenic proteins.

FESEM images (i) of the 3 nanopellets (NPs):

(A) IPNV-VP2^{NP},

(B) VHSV-G-frg16^{NP}

(C) VNNV-C^{NP};

with corresponding size distribution histograms (n=120) for (ii) width (nm) and (iii) length (nm). Note there is no histogram (iii) for (C), as these NPs were amorphic in length.

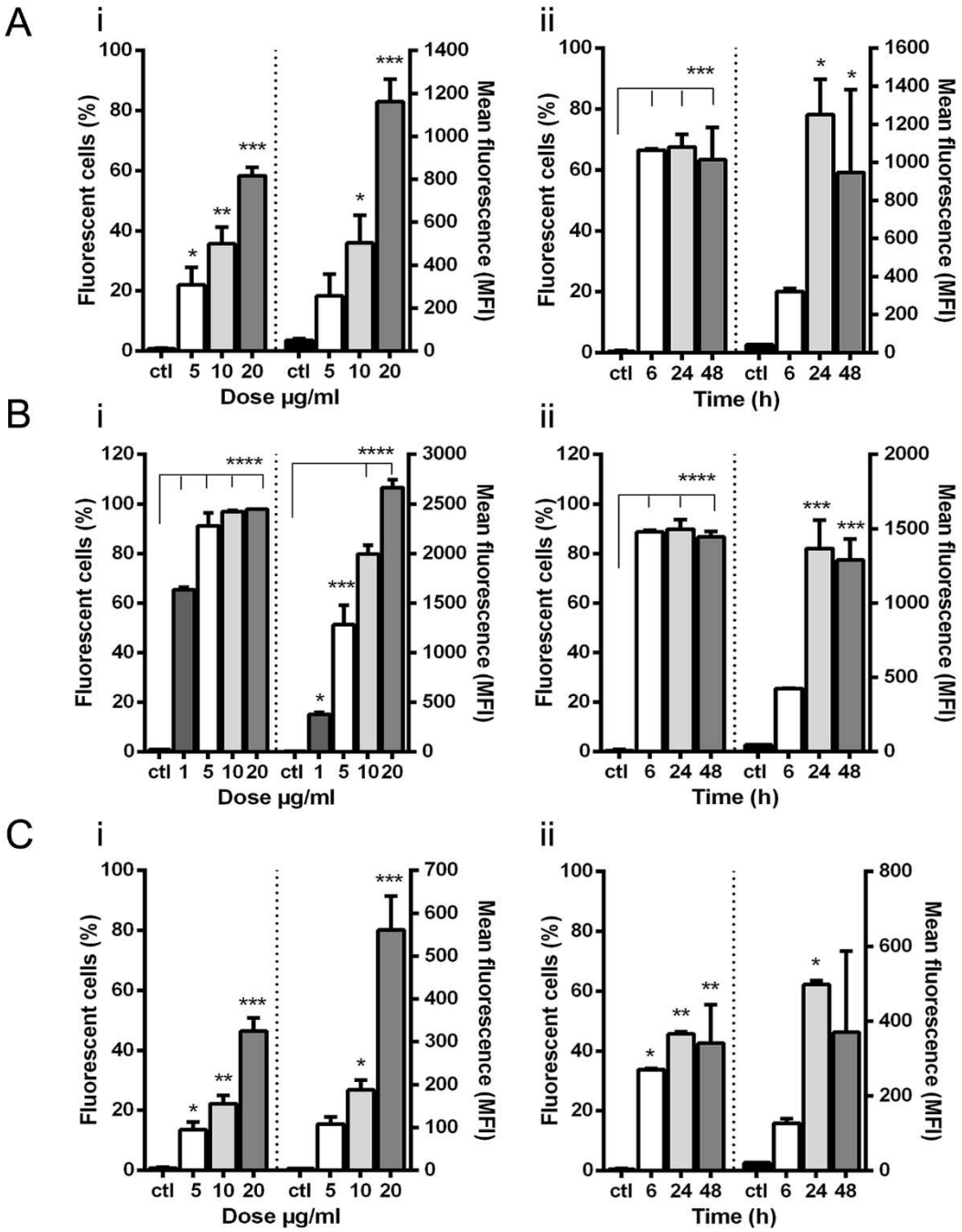


FIGURE 4: Uptake of viral NPs by ZFL. Fluorescently labelled NPs (A) IPNV-VP2^{NP}, (B) VHSV-G-frg16^{NP}, and (C) VNNV-C^{NP} were added to ZFL. Control (ctl) was ZFL without NPs. (i) **Dose response**. Cells incubated for 12 h with NPs (A) and (C) at 5-20 µg/mL, and (B) at 1-20 µg/mL in duplicate. (ii) **Time course.** NPs added to cells at 10 µg/mL (A) and (C), and 5 µg/mL (B) in duplicate and incubated for 6-48 h. Differences between means were analyzed by a 1 way ANOVA with Dunnett's multiple comparisons test, treatments versus control. Significance levels *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

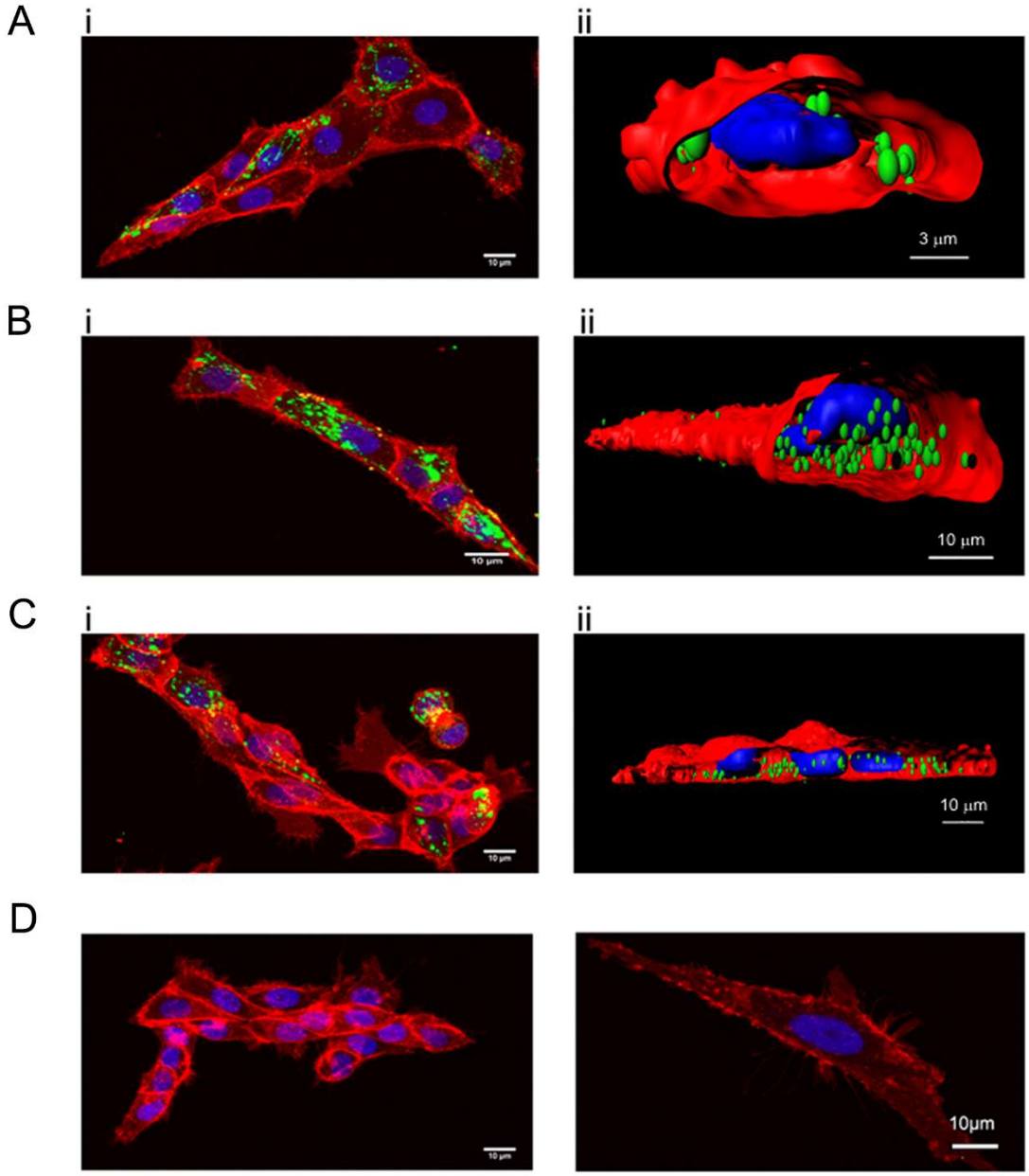


FIGURE 5: Confocal microscopy (i) and **digitalized image** (z-stacks) (ii) of ZFL cells after 14 h incubation with NPs: 20 µg/mL (A) IPNV-VP2^{NP} and (C) VNNV-C^{NP} and 10 µg/mL (B) VHSV-G-frg16^{NP}. NPs are green, cell membrane red and nuclei blue. (D) Control confocal images of ZFL without NPs.

Figure 5 shows confocal microscopy images of NP uptake by ZFL after O/N incubation. In the case of IPNV-VP2^{NP} and VNNV-C^{NP}, **Figure 5A** and **5C**, there are cells which have taken up a lot of NP, but others which have very few or no NPs. This is consistent with the cytometry results in which the maximum percentage of fluorescent cells taking up these particles O/N, at the same dose as the confocal experiments (20 µg/mL), were ~60 % and 50 % respectively (**Figure 4A** and **4C** (i)). There are therefore some cells which do not up take IPNV-VP2^{NP} and VNNV-C^{NP} under these conditions. In contrast, all cells we observed in confocal microscopy had taken up VHSV-

G-frg16^{NP} in large quantities **Figure 5B**. This concords with O/N cytometry results at the same dose (10 µg/mL), which reached 100 % fluorescent cells (**Figure 4B** (i)). The digitalized z-stack images (**Figure 5 A-C** (ii)) clearly show all 3 NPs have been internalized by the cells. For VHSV-G-frg16^{NP} some particles are also visibly embedded in the membrane and numerous NPs are inside the cell (**Figure 5B** (ii)). Imaris imaging software allows estimating the number of nanoparticles per cell. In a small sample, the NPs/ZFL cell were as follows (mean and SD): IPNV-VP2^{NP}, 50 ± 19 NPs/cell and 67 % of cells counted had NPs (n=9); VNNV-C^{NP}, 57 ± 31 NPs/cell and 65 % of cells had NPs (n=20); VHSV-G-frg16^{NP}, 88 ± 45 NPs/cell and 100 % of cells had NPs (n= 11).

4.2.1 Cytotoxicity test in ZFL

To check if the NPs had cytotoxic or cytostatic effects MTT assays were performed in ZFL. The cells incubated with 10, 20 and 50 µg/mL of each NP for 14 h showed no significant difference in survival between control and any treatment group. This indicated that none of the NPs were significantly affecting viability **Figure 6**. Note also in the *in vivo* oral gavage experiments in zebrafish, animals were kept up to 48 h post-intubation (see section 3.3.2) and fish showed no signs of malaise. In fact, in other work we have previously injected up to 300 µg/fish of nanostructured TNF-α and maintained the animals for 30 days with no signs of any deleterious effects (13).

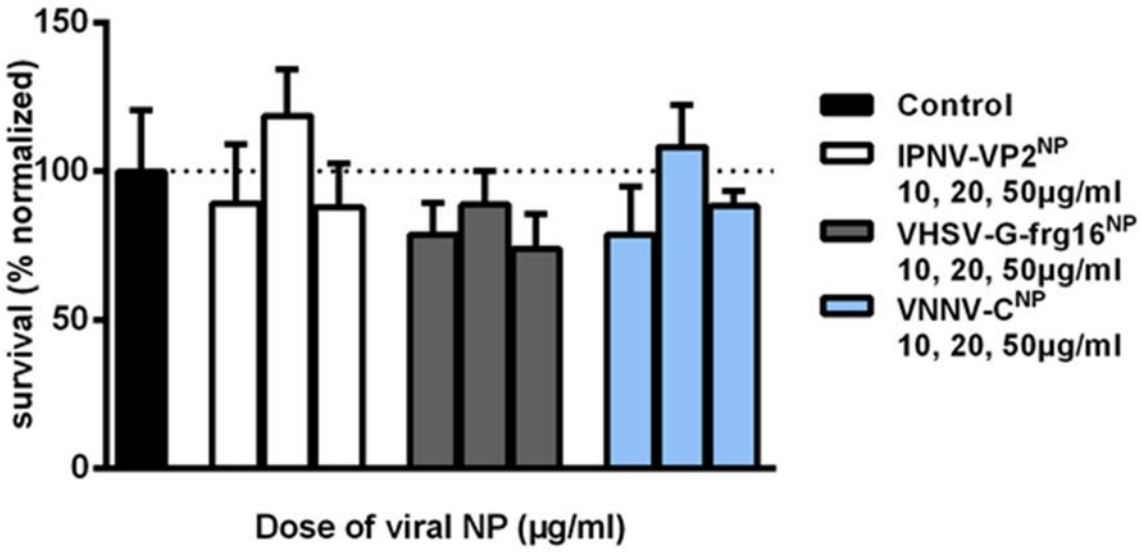


FIGURE 6: MTT assay of NP cytotoxicity in ZFL. ZFL stimulated with viral NPs at 10, 20 and 50 µg/mL in triplicate for 14 h at 28 ºC. Positive control, without NP. Negative control, cells treated with 1 % Triton. After MTT treatment, absorbance at 550 nm was normalized with controls. The experiment was repeated twice. Data analysis: one-way ANOVA with Dunnett's multiple comparisons test between each treatment and control mean, at significance level $p < 0.05$. No treatment group was significantly different from control.

4.3 Gene expression analysis in ZFL stimulated with NPs

To see whether the NPs could elicit an innate immune response in line with that provoked by viral infection, ZFL were stimulated with the 3 viral NPs O/N at 10 µg/mL for IPNV-VP2^{NP} and VNNV-C^{NP} and 5 µg/mL for VHSV-G-frg16^{NP}. We used a half-dose for VHSV-G-frg16^{NP} compared to the other NPs, given that uptake of this nanoparticle in ZFL had been greater than the others, even at this lower dose (**Figure 4B**). We used poly (I:C) (25 µg/mL) as a viral dsRNA mimic, and iRFP^{NP} (10 µg/mL) as a control NP made with an immunogenically irrelevant protein. Gene expression of 6 gene markers of the innate immune response to viral infection were tested by qPCR (**Figure 7**). For all genes tested there was a remarkable similarity in the response to poly (I:C) and VNNV-C^{NP}, significantly different from the untreated control. For *vig1* and *gig2* the upregulation was several thousand-fold for both treatments. For *stat1b* the mean fold change (± SD) was 178 ± 32 for poly (I:C) stimulated cells and 160 ± 41 for ZFL stimulated with VNNV-C^{NP}. *Mx* and *irf7* were upregulated between 27 ± 3 to 39 ± 3 fold by both treatments, while *ccl4* was upregulated 17 ± 4 fold and 23 ± 8 fold by poly (I:C) and VNNV-C^{NP} respectively.

For the other 2 viral NPs, the fold change in gene expression was positive but much lower. IPNV-VP2^{NP} elicited a statistically significant upregulation for all genes except *ccl4*, ranging from 9 ± 2.4 fold for *vig1* to 2 ± 0.5 fold for *irf7*. VHSV-G-frg16^{NP} only elicited a significant upregulation for 3 of the genes tested: *gig2,* 7 ± 2.5 fold; *stat1b,* 2.5 ± 1.0 fold and *mx* 1.5 ± 0.2 fold. iRFP^{NP} was significantly, though slightly upregulated for 2 of the genes tested: 2 ± 0.8 fold and 1.7 ± 0.4 for *irf7* and *stat1b* respectively.

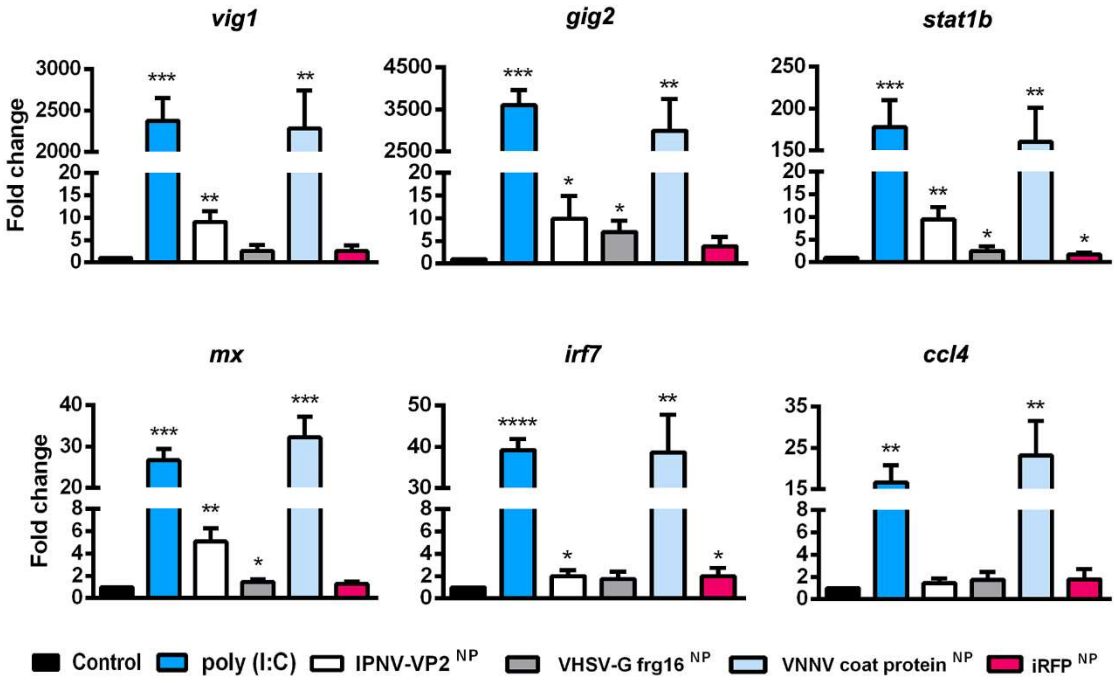


FIGURE 7: Gene expression analysis in ZFL stimulated with NPs. Cells incubated for 14 h with: untreated control (black), poly (I:C) 25 µg/mL as a positive control (blue), IPNV-VP2^{NP} 10 µg/mL (white), VHSV-G-frg16^{NP} 5 µg/mL (grey), VNNV-C^{NP}10 µg/mL (light blue) and iRFP^{NP} 10µg/mL as an immunogenically irrelevant NP control (red). Samples are from 2 independent experiments. Data are mean ± SD (n = 4). Gene expression was determined by qPCR with 3 technical replicates. Differences between each treatment mean & control were analyzed by unpaired one-sided t-tests with Welch's correction for unequal variances. Significance levels *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

4.4 Gene expression analysis in RT-HKM stimulated with salmonid viral NPs

As the innate immune response to VHSV-G-frg16^{NP} had been weak in ZFL except for *gig2*, we decided to test the NP-based stimulus in RT-HKM primary cultures. Using macrophages from trout, a natural host for VHSV and IPNV, would provide more pertinent *in vitro* data for the two NPs formed by salmonid viral antigenic proteins. We therefore incubated RT-HKM with IPNV-VP2^{NP} and VHSV-G-frg16^{NP} as well as poly (I:C) and iRFP^{NP} controls all at 10 µg/ml. Genes tested included *vig1*, *mx* and *ifit5* which are relevant markers of VHSV infection (28), as well as *mda5* and *ccl4*. For all genes tested both IPNV-VP2^{NP} and VHSV-G-frg16^{NP} evoked upregulation, significantly different from the untreated control (**Figure 8**) as follows: *vig1* 5.6 ± 4.1 and 5.1 ± 3.2 fold for IPNV-VP2^{NP} and VHSV-G-frg16^{NP} respectively; continuing in that order *ifit5* 7.1 ± 1.7 and 6.9 ± 1.6; *ccl4* 16.9 ± 10.8 and 16.2 ± 10.2; *mx* 2.6 ± 1.4 and 3.3 ± 1.1; *mda5* 3.0 ± 1.8 and 3.3 ± 1.2. For all genes tested, the poly (I:C) positive control elicited higher upregulation than the NPs, but the difference was not as great as that seen in ZFL. Note here, the poly (I:C) dose

used was the same (10 µg/ml) as for the NPs, whereas in ZFL we used 25 µg/ml (30). The most similar response to stimulus with poly (I:C) and to the NPs was seen for *mda5* which was upregulated 7.1 ± 1.3 with poly (I:C) treatment. Treatment with the control nanoparticle, iRFP^{NP}, only significantly upregulated 1 gene very weakly, *ifit5* 1.8 ± 0.4 fold.

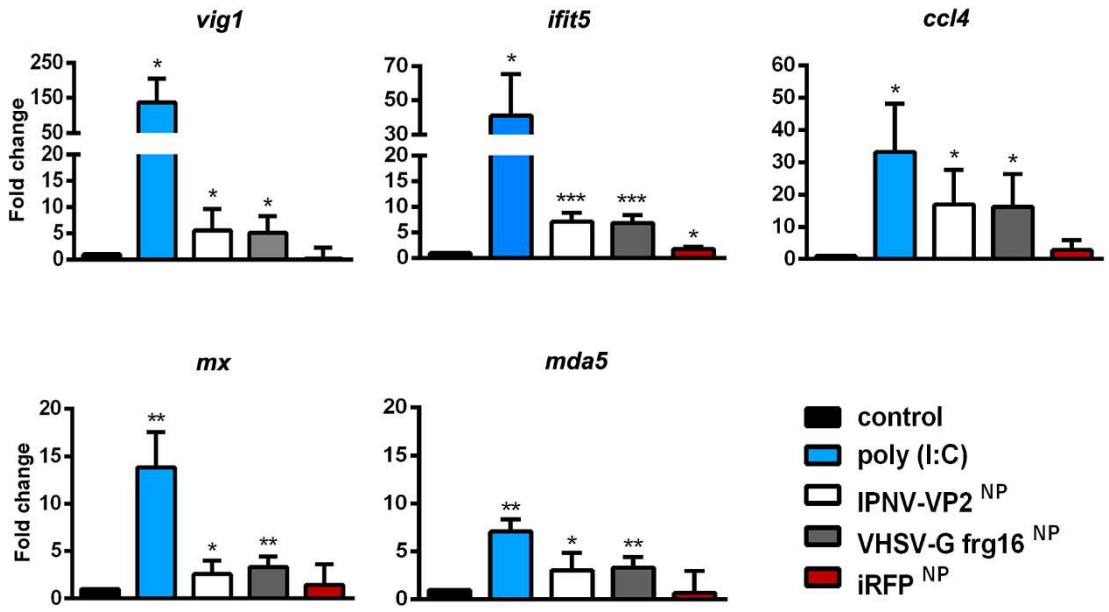


FIGURE 8: Gene expression analysis in RT-HKM stimulated with salmonid viral NPs. Cells incubated for 15 h with: untreated control (black), poly (I:C) 10 µg/mL as a positive control (blue), IPNV-VP2^{NP} 10 µg/mL (white), VHSV-G-frg16^{NP} 10 µg/mL (grey) and iRFP^{NP} 10 µg/mL as an immunogenically irrelevant NP control (red). Samples are from 2 independent experiments. Data are mean ± SD (n = 4). Gene expression was determined by qPCR with 3 technical replicates. Differences between each treatment mean & control were analyzed by unpaired one-sided t-tests with Welch's correction for unequal variances. Significance levels *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

4.5 Uptake of NPs by zebrafish via oral gavage

In preliminary *in vivo* experiments, adult zebrafish (n=3) were orally intubated with viral NPs at 20 and 50 µg/fish and sampled at 6, 24 and 48 h. By 24 h the percentage of fluorescent cells had dropped by approximately 50% compared to 6 h and had dropped further by 48 h, indicating early uptake of the NPs *in vivo* (data not shown). Hence the intubation experiments with larger numbers of fish, reported here (**Figure 9**), were done at a short time interval of 5 h. Adult zebrafish were able to take up the 3 viral NPs into gut cells when administered orally via intubation at 20 µg/fish.

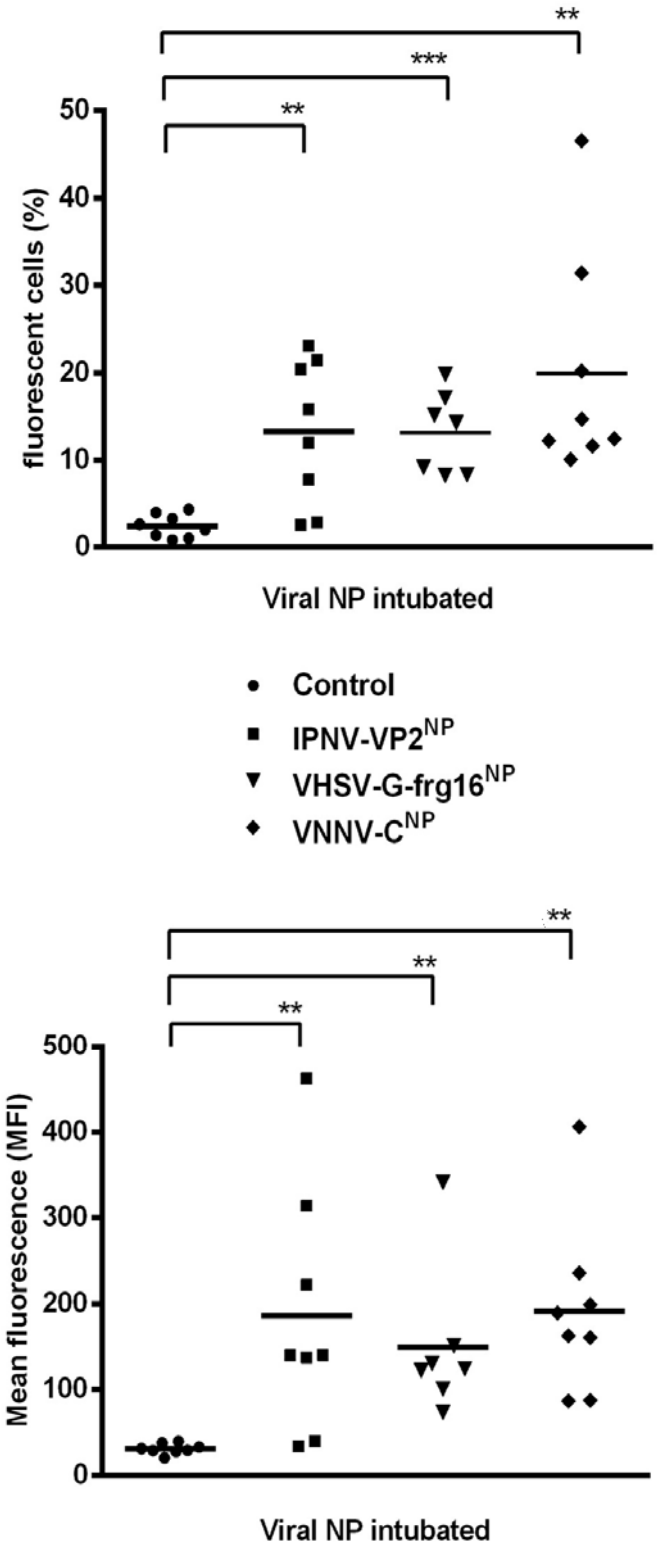


FIGURE 9: Uptake of NPs by zebrafish via intubation.

Adult zebrafish were intubated with 20 µg/fish of each fluorescently labelled NP in 30 µL PBS for 5 h (n=8), then intestine cells were sampled for cytometry.

● Control fish: 30 µL PBS intubated without NP. Each point represents data from 1 fish intubated with ■ IPNV-VP2^{NP}, ▼ VHSV-G-frg16^{NP}, or ♦ VNNV-C^{NP}. Horizontal bars are the means. Differences between the mean of each treatment group & control were analyzed by an unpaired one-sided t-test with Welch's correction for unequal variances. Significance levels *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

In **Figure 9**, upper graph, for IPNV-VP2^{NP}, 75% of the fish intubated had taken up NP by 5 h, while for VHSV-G-frg16^{NP} and VNNV-C^{NP}, 100 % of the fish intubated internalized the NPs (n=8). Range and mean of the percentage of fluorescent cells (10,000 events) were: range 0-23 %, mean 13 % for IPNV-VP2^{NP}, range 8-19 %, mean 13 % for VHSV-G-frg16^{NP}, and range 10-47 %, mean 20 % for VNNV-C^{NP}. The mean fluorescence intensity (MFI) results (**Figure 9** lower graph) in general clustered around the average for each group, being 186, 151 and 191 for IPNV-VP2^{NP}, VHSV-G-frg16^{NP} and VNNV-C^{NP} respectively. Note the fluorescence labelling efficiency with Atto-488 NHS was lower for VHSV-G-frg16^{NP} compared to the other 2 NPs (See **Figure 2**). This explains the lower average MFI in intestine cells which had taken up VHSV-G-frg16^{NP}.

5. Discussion

The thrust of our work is to seek a safe and effective, but eminently practical solution for fish vaccination in the long term. To this purpose, we have successfully produced 3 viral antigenic proteins in *E. coli* as IBs. The production of these "nanopellets" (NPs) followed a simple, fully scalable, batch culture procedure in *E. coli*, with isolation by mechanical and enzymatic methods. This is a more straightforward, less costly approach than that required to produce VLPs (8), or purified soluble recombinant proteins and avoids safety issues raised regarding DNA vaccines. Concerning biocompatibility, the NPs were not toxic to ZFL cells, nor were there any signs of malaise in adult zebrafish intubated with up to 50 µg/fish for 48 h. In previous work, we have injected IBs at up to 300 µg/zebrafish and maintained the animals for 30 days with no signs of any deleterious effects (13). We therefore consider the recombinant protein NPs are innocuous to fish. Having successfully produced the NPs, we wished to address two critical questions at this stage: Could the NPs be taken up in fish? And, would an initial immune response be evoked, given the importance of the innate immune response in establishing an effective adaptive immune response to vaccination (31)?

As regards uptake, an advantage of IBs is that the amyloid scaffold can protect the functional protein from degradation while passing through the low pH of the gastro-intestinal tract. We have already successfully tested other NPs resistance at pH 2.5 and uptake in intubated trout (13). The scaffold itself is resistant to proteinase K digestion but represents approximately 20 % of the protein in the structure (32), leaving a considerable amount of functional protein to be released slowly within the organism. Here we tested first, uptake *in vitro* in ZFL and then *in vivo* in zebrafish via intubation. In ZFL all three NPs were taken up O/N, achieving ≥ 50 % of the cells sampled. VHSV-G-frg16^{NP} uptake was strikingly efficient even at 6 h, the earliest time point tested. The abundant uptake of VHSV-G-frg16^{NP} by ZFL was corroborated by the confocal microscopy results. For the two other NPs, uptake was also high in susceptible cells, but not all cells had internalized the particles. The VHSV-G-frg16^{NP} construct contains an arginine-glycine-aspartic acid (RGD) tripeptide integrin binding site (18, 19), not present in IPNV-VP2^{NP} nor VNNV-C^{NP}. RGD-binding integrins are known receptors or coreceptors for certain viruses (33). In addition, in experiments on IB uptake in HeLa cells, an IB with the RGD site mutated to RGE was internalized significantly less than that with RGD (25). We thus hypothesized the RGD site in VHSV-G-frg16^{NP} may be facilitating IB uptake in ZFL.

The *in vivo* uptake results in zebrafish were also encouraging. The three NPs were able to be taken up by almost all fish tested via the intestine in a matter of hours. The zebrafish gut is composed of intestinal epithelial cells, goblet cells, smooth muscle cells (see Figure 1A in (34)) and immune cells also known as gut-associated lymphoid tissue (GALT). The GALT is particularly important

because it is the main immune tissue involved in the uptake and processing of orally administrated antigens (10). We found an average of 13 %, 13 % and 20 % of cells had taken up IPNV-VP2^{NP}, VHSV-G-frg16^{NP} and VNNV-C^{NP} respectively, 5 h after oral administration of a single dose. We do not know which specific cell type is taking up the viral NPs but in previous work we have shown that cytokine-made NPs can be found in the lamina propria (midgut) and in the villi apex where lymphoid cells are located (13).

The development of the zebrafish oral gavage method used should also be noted. We are able to successfully administer up to 30 µL, to fish of mean weight 0.9 ± 0.2 g simply and quickly, without injuring the animals. The fact that fish were able to take up the NPs via the oral route is crucial as a proof of concept for a strategy to evoke mucosal immune stimulus. Nevertheless, while antigen uptake is a point in favor, it is by no means a guarantee of an immune response, as the gut environment is highly tolerogenic. This is one of the main challenges in oral vaccine development, which we will need to face further down the pipeline (10, 35).

At this stage, the other issue studied here regarding the potential use of NPs was whether they could evoke an anti-viral innate immune response. We therefore stimulated ZFL cells with the three viral antigen NPs and the control iRFP^{NP} and checked expression of innate immune gene markers of viral infection: IFN stimulated genes (36) including transcription factors *irf7* and *stat1b* and genes encoding anti-viral peptides *mx* and *viperin* (*vig1*) (37), as well as *gig2* and chemokine *ccl4*. The viral dsRNA mimic, poly (I:C), was used as a positive control as it mounts an anti-viral response in zebrafish (30) among other species, and as such is being tested as a potential fish vaccine adjuvant (38). The results for VNNV-C^{NP} were particularly promising. All six genes tested were highly upregulated, attaining similar levels to those obtained with poly (I:C). IPNV-VP2^{NP} also caused significant but much lower up regulation, while VHSV-G-frg16^{NP} only upregulated 3 of the genes at lower levels. Upregulation by the control NP, iRFP^{NP} was slight or negligible. The poly (I:C) positive control was not conceived for direct quantitative comparison, as it mimics nucleic acid, not protein. For this reason, we were surprised that the upregulation of the innate immune genes tested appeared so similar, between VNNV-C^{NP} and poly (I:C). Multiple activation pathways are triggered by viral infection (39), but we had not expected such a comparable profile of gene upregulation by the recombinant protein and the viral dsRNA mimic. Apparently, we had achieved an innate anti-viral response in full swing, by two quite different stimuli.

Indeed, the role of viral capsid proteins in innate immune stimulus is starting to be elucidated by research in mammalian systems. It appears that innate immune activation can be mediated by recognizing the intrinsic order of capsid structure. For instance, TRIM5 has been reported as a pattern recognition receptor, specific for retrovirus capsid lattice (40). Further, Toll-like-receptor-

2 (TLR2) has recently been shown to respond to the multi-subunit arrangement of viral capsids, independent of amino acid sequence, or specific morphology. Rather, stimulus relies on repeating protein subunits, as a conserved common denominator across viral capsids (41). We do not know how well our NPs fit into this descriptor, but IBs are entities composed of repeated subunits in an ordered nanostructure. Fourier transform infrared (FT-IR) micro-spectroscopy shows that IBs are proteins with native-like structure entrapped in densely packed intermolecular β-sheet bridges (42). The relative amount of native-like protein can differ with production conditions. Out of interest, we checked crystallography data from a VLP of Grouper nervous necrosis virus (GNNV) (43), another marine betanodavirus. The self-assembled particle size is typical of the *Nodaviridae* 30-35 nm, and the shell domain has the common viral capsid protein jelly-roll structure with 8 β strands forming 2 antiparallel sheets (44). Our VNNV-C^{NP} is considerably larger (~ 420 nm) than the VLP and we do not know the 3D structure further than the order inferred from the FESEM images. We also do not know if there is self-assembly of the native-like viral capsid protein as it emerges from the IB scaffold. Nevertheless, our results imply that this NP triggered an innate immune response in ZFL cells as if it were a virus.

It should also be pointed out that the NPs, while made mainly of viral protein subunits, contain low amounts of bacterial nucleic acids, peptidoglycan and lipopolysaccharide (14). The non-relevant immune control, $iRFP^{NP}$ also has these contaminants but was a poor stimulator of the antiviral response both in ZFL and HKM cells. This does not preclude stimulus of other genes. In fact, in prior work, when $iRFP^{NP}$ was injected in zebrafish and a challenge with *Pseudomonas aeruginosa* was performed, there was significant survival of treated fish compared to control. The protection was presumably due to stimulus evoked by these contaminants (14).

Regarding IPNV-$VP2^{NP}$ and VHSV-G-$frg16^{NP}$, the important consideration for our purposes was that the NPs could stimulate the chosen viral response gene markers, more than the size of the effect. In this vein, we were concerned that VHSV-G-$frg16^{NP}$ had not produced stimulus in several of the genes tested in ZFL. It should be kept in mind that this NP construct is not the whole antigenic protein, in contrast to the other NPs, but it has antigenic epitopes including Mx inducing sites (18). Given that tropism might be a significant factor, we tested the expression of viral response gene markers, induced by IPNV-$VP2^{NP}$ and VHSV-G-$frg16^{NP}$ in RT-HKM primary cultures, as trout is a natural host for IPNV and VHSV. In these experiments, we included *ifit5* (28) and *mda5* (36) an IFN induced gene and a dsRNA receptor belonging to the RIG-1-like receptor family, respectively. In this case we got significant stimulus of all the gene markers, at a similar level for both NPs.

Summarizing, we have produced 3 recombinant viral antigenic proteins as nanostructured biomaterials with view to use in orally delivered prophylaxis. The methodology employed is

straightforward, cheap and fully scalable. These “nanopellets” are successfully taken up *in vitro* in ZFL and *in vivo* in zebrafish via oral administration. They stimulate an anti-viral innate immune response both in ZFL and RT-HKM cells. They therefore are candidates for immunostimulants. On the road to vaccine development, the next essential steps are to run protection studies and to demonstrate the raising of antigen-specific antibodies in target fish species. We are keen to further explore their potential.

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CHAPTER 2

IN VIVO STUDIES IN FARMED FISH MODELS (1): VNNV-C^{NP} and Senegalese sole *(Solea senegalensis)*

Nanostructured recombinant protein particles raise specific antibodies against the VNNV coat protein *in vivo* via i.p. injection and a novel oral gavage method.

1. Abstract

Viral nervous necrosis virus (VNNV) reassortant strains RGNNV/SJNNV have emerged as a potent threat to the Mediterranean marine aquaculture industry, causing viral encephalopathy and retinopathy (VER) in Senegalese sole (*Solea senegalensis*), gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). We have designed a cheap and practical vaccine strategy using bacterial inclusion bodies made of the coat protein of a virulent reassortant strain of this betanodavirus. Here we report our nanostructured recombinant protein nanoparticles, VNNV-C^{NP}, raise specific antibodies *in vivo* against the VNNV coat protein, as a primary surrogate of protection. We test two administration routes in Senegalese sole juveniles: intraperitoneal injection and oral gavage. The oral administration is via a novel method which delivers precise doses to test oral vaccines or other compounds relevant for intestinal uptake studies. Results from ELISA show the production of anti-VNNV coat protein immunoglobulins via both routes and two doses, with a substantial specific antibody expansion 30 days post booster in the injected group. We include gene expression data for *cd8-α, cd4, IgM, IgT* and *arg2* to provide insight into the dynamics of the adaptive immune response via the different administration routes. The strong antibody response elicited *in vivo,* as well as the lack of any signs of macro-toxicity over the 6-week study period using N=100 young fish, evidences the potential of this nanoparticle as a vaccine candidate.

2. Introduction

Viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN), is a serious infectious disease affecting a wide array of fish species worldwide, except in South America. Wild and farmed marine fish are susceptible including some relevant commercial species such as Senegalese sole (*Solea senegalensis*), European sea bass (*Dicentrarchus labrax*), Asian sea bass or barramundi (*Lates calcarifer*) and groupers (*Ephinephelus spp*.)(1, 2). The causative agent, viral nervous necrosis virus (VNNV), is a non-enveloped, small (~30 nm diameter), icosahedral virus with a bi-segmented single-stranded (+) RNA genome. This *Betanodavirus* has tropism for the nervous system and fish with VER present necrosis and vacuolation in the brain, retina and spinal cord (3). Clinical signs include abnormal swimming, spasms, changes in colouration, anorexia and lethargy (4). Mortality is particularly high in larvae and juveniles and the virus can be transmitted vertically through broodstock (5) as well as horizontally through the water body from infected fish, asymptomatic carriers and infection survivors or carried in invertebrates used as feed for marine fish larvae; reviewed in (1, 2).

Husbandry practices such as feeding small captured wild fish and squid to farmed species could also contribute to horizontal transmission (6).

RNA1 of the genome encodes RNA-dependent RNA polymerase and is also involved in determining temperature sensitivity for viral replication (7). From the 3'end of RNA1, sub-genomic RNA3 is transcribed during viral replication in infected cells, encoding proteins B1 and B2, the latter being a RNA silencing-suppression protein that facilitates accumulation of intracellular viral RNA1 (8). RNA2 encodes the capsid, or coat protein and contains a variable region which was used to classify four different genotypes based on phylogenetic analysis (9): red grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV) and barfin flounder nervous necrosis virus (BFNNV). These are the 4 species of *Betanodavirus* which currently feature on the International Committee on Taxonomy of Viruses (ICTV) website https://talk.ictvonline.org/taxonomy/ (consulted 19.3.2019). It is the coat protein which plays a key role in infectivity (10). **Figure 1** shows brain tissue of grouper larvae infected with RGNNV and a schema of the segmented +ssRNA genome of fish nodaviruses.

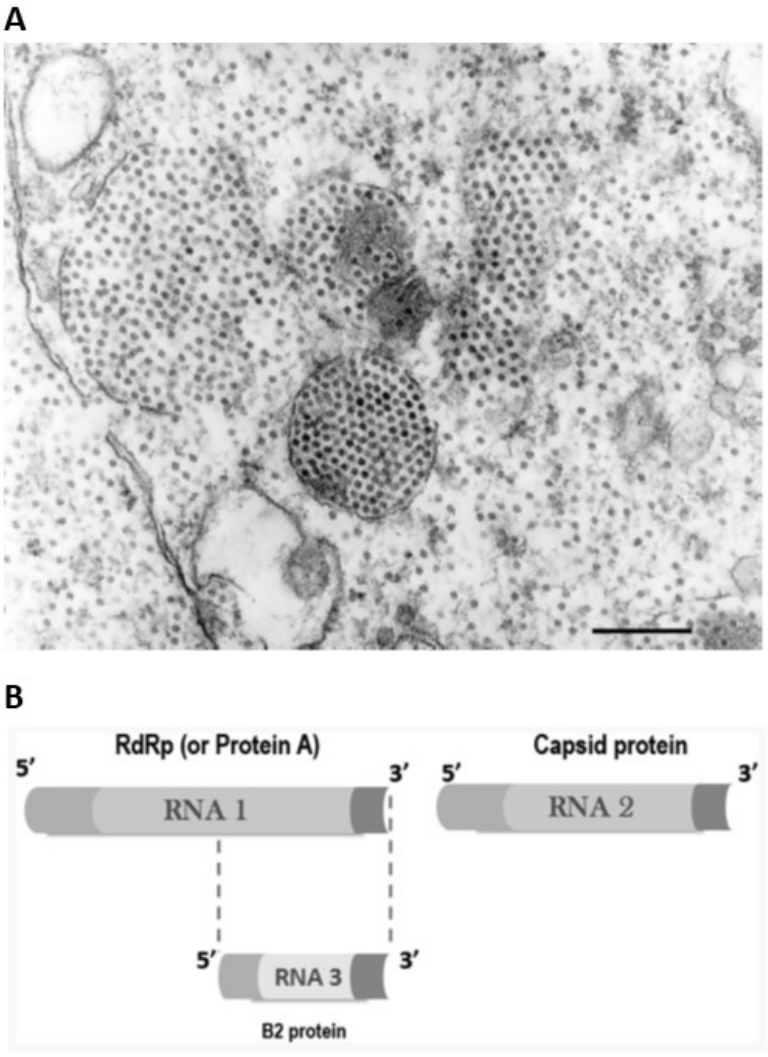

FIGURE 1: Betanodavirus

A. VNNV infected tissue

RGNNV particles in the brain tissue of grouper larvae. TEM (Transmission Electron Microscopy) image bar = 400 nm. Viral particle typical size is ~30 nm

B. VNNV genome

General organization of the betanodavirus genome. Genomic +ssRNA1 & 2; & subgenomic RNA3. RdRp = RNA dependent RNA polymerase.

Both images reprinted with permission from (11)

Recently in the Mediterranean, reassortant strains RGNNV/SJNNV have emerged, showing high virulence in Senegalese sole (12, 13). Reasssortants also cause mortality and clinical signs of VER in farmed European sea bass, though the disease is not as severe nor as widespread as that caused by the RGNNV strain (13, 14). Worryingly, in 2014-16 hatcheries in Southern Europe started reporting mass mortalities in gilthead sea bream (*Sparus aurata*) larvae which tested positive for RGNNV/SJNNV (15). Until that time sea bream had been considered resistant to NNV, though they could be asymptomatic carriers. In addition, turbot (*Scophthalmus maximus*) juveniles have been shown to be susceptible to the RGNNV/SJNNV reassortant (SpSs-IAusc160.03) isolated from diseased Senegalese sole (16). Considering the farming of Senegalese sole is in close proximity to turbot in Galicia (North West Spain), temperatures of 18 ºC are permissive, and interspecies transmission of betanodavirus has been previously reported (17), this new threat to the aquaculture industry is looming on many fronts.

The first commercial vaccine against RGNNV in Europe has just been released by Pharmaq, ALPHA JECT micro®1Noda. It is a formaldehyde-inactivated culture of RGNNV strain ALV1107 in a liquid paraffin adjuvant for intraperitoneal (i.p.) injection to European sea bass of minimum weight 12 g https://www.pharmaq.no/products/injectable/mediterranean/. While this may be very suitable for broodstock to avoid vertical transmission, infection otherwise occurs primarily at larval and young stages where i.p. vaccination is not feasible (4). Alternatively, if juveniles are vaccinated i.p. this is very labour intensive and costly. Currently, we do not have any data about the protective effects of this vaccine against reassortant RGNNV/SJNNV strains, nor its application in any other species than *D. labrax*. However, cross-protection between VNNV genotypes has so far not been evidenced (1).

As regards other vaccine developments, apart from inactivated virus, different strategies such as viral like particles (VLPs), DNA vaccines, and recombinant capsid proteins injected i.p. or to muscle (i.m.), have been explored. Using these strategies, protection against an RGNNV or a SJNNV challenge, and the raising of neutralizing antibodies has been, for the most part, successfully demonstrated in several species of farmed fish affected by VER in Europe (sea bass and turbot) or in Asia and Oceania (barramundi and groupers). This has been extensively reviewed in (1). The onus now is to develop oral or immersion vaccines in line with the practical needs of the industry. Recent approaches are using VLPs mixed in the feed or by bath immersion to orange spotted grouper (*Epinephelus coioides*) larvae (18) or administration of VLPs by oral gavage to convict grouper juveniles (*Epinephelus septemfasciatus*) (19), compared with delivery by injection, achieving encouraging results.

Here we focus on a new vaccine strategy for the reassortant RGNNV/SJNNV strain with view to providing a practical tool to combat VER in Senegalese sole, gilthead sea bream and turbot. To

this purpose we have produced bacteria inclusion bodies (IBs) in *Escherichia coli* made of the coat protein of the betanoadvirus reassortant strain (SpSs-IAusc160.03). IBs are biologically active, non-toxic protein nanoparticles which have a propensity to cross cell membranes and can serve as protein releasing agents (20). They are stable *in vivo* without encapsulation and are cheap and easy to produce as recombinant protein nanoparticles (21). In addition, they carry immunostimulants for fish in the form of remnants of bacterial lipopolysaccharide (LPS), peptidoglycans and nucleic acids (22). We have already demonstrated that our RGNNV/SJNNV coat protein "nanopellet" (NP), VNNV-C^{NP}, induces a strong, innate anti-viral immune response *in vitro* in zebrafish liver cell line (ZFL), as well as being readily taken up *in vivo* through the zebrafish intestine after oral administration (23). We now test the capacity of the nanoparticle to elicit specific antibodies against the VNNV coat protein of the same strain in juvenile Senegalese sole, as a surrogate of protection (24). We compare i.p. injection and the oral route using a novel oral gavage method to sole that permits delivery of precise amounts of nanoparticle to young fish.

3. Materials and Methods

3.1 Fish

S. senegalensis larvae (pre-mouth opening) were purchased from Cupimar (Cadiz, Spain) and raised at IFAPA, El Toruño (Instituto de Investigación y Formación Agraria y Pesquera), Cádiz, in an open circulation system of filtered sea water. For all manipulations fish were anesthetized using 99% 2-phenoxyethanol (Sigma) 0.4 mL/L. (sedation 0.1 mL/L, euthanasia 0.6 mL/L). Four days pre-administration, 100 fish, average weight 15.21 ± 1.93 g, were colour coded by injecting a thin line of dye (Visible Implant Elastomer, Northwest Marine Tech) under the ventral skin: 50 green at the proximal end for injection, 50 red at the distal end for oral gavage (see **Figure 2**). Fish were moved to two tanks for 3 days adaptation. Throughout the experiment they were given commercial fish feed daily (1.5 mm diameter, 2% of biomass), except for 24 hours prior to administration or sampling.

3.2 VNNV coat protein production

3.2.1. VNNV-C^{NP} nanoparticle

The protein nanoparticle VNNV-C^{NP} contains the VNNV coat protein from the Iberian betanodavirus isolate (strain SpSs-IAusc160.03), NCBI GenBank, accession no: NC_024493.1. This is a reassortant RGNNV/ SJNNV strain (12). The cloning strategy included a C terminal Histag. Production as bacterial inclusion bodies in *E.coli* , physical characterization, the innate anti-viral immune response to the nanoparticle *in vitro* in zebrafish liver cell line (ZFL), as well as uptake *in vivo* by zebrafish is reported in (23), which is Chapter 1 of this thesis.

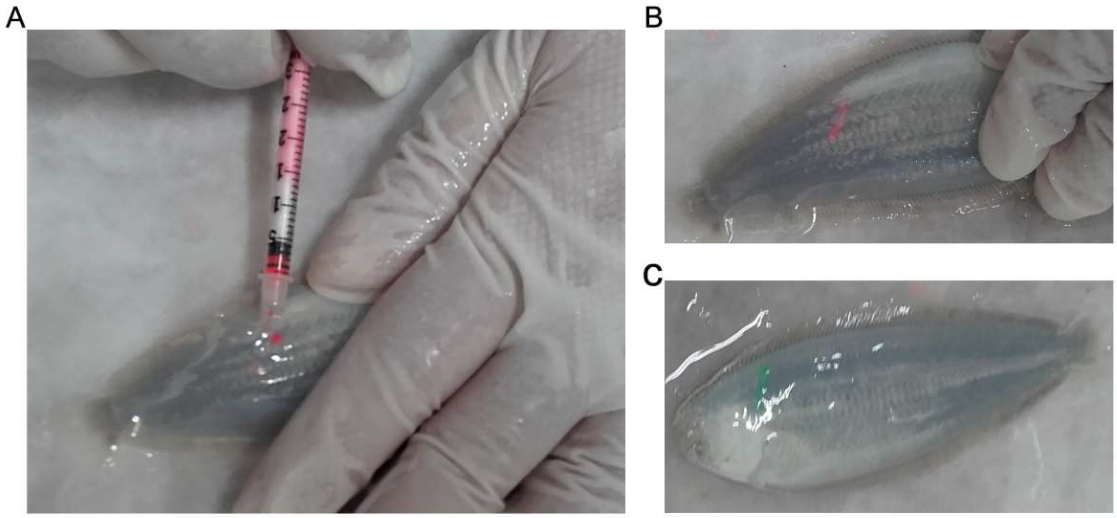

FIGURE 2: Colour coding of sole. A. Fish were colour coded by injecting a thin line of dye (Visible Implant Elastomer) under the ventral skin. **B**. Red at the distal end for fish destined for oral gavage **C**. Green at the proximal end for fish to be injected i.p.

3.2.2 Soluble VNNV for ELISA

With the same clone used to make bacterial inclusion bodies (VNNV-C^{NP}), we were able to produce soluble VNNV coat proteinHis6 by changing the culturing conditions. *E. coli* was cultured at 37 ºC in LB with ampicillin (Sigma) at 100 µg/mL until OD550 nm reached 0.5–0.8, as usual. Then protein expression was induced with 1 mM IPTG (Panreac) and the culturing temperature was reduced to 16 ºC. Growth continued slowly overnight (O/N). To confirm production of soluble VNNV coat protein His6, an equivalent number of cells from a sample of the cultures (pre and post IPTG O/N) were collected by centrifugation at 5000 x *g* for 15 min at 4 ºC and resuspended in 1mL PBS with protease inhibitor (cOmplete-EDTA free, Roche); then sonicated on ice 3 x 1 min at 10% 0.5 sec on/0.5 sec off and centrifuged at 15000 x *g* for 15 min at 4 ºC to separate the soluble protein fraction (supernatant) from the insoluble (pellet). Aliquots were run on a western blot and the protein was detected using an anti-His-tag antibody (Genscript A00186-100), see **Figure 3A**. For use in enzyme-linked immunosorbent assays (ELISAs), soluble protein was purified by fast protein liquid chromatography (Akta Purifier 10, GE Healthcare) via the C terminal Histag. Purification was performed using 0.1M $NiCl_2$ on a 1 mL HiTrap Chelating HP column (GE Healthcare), eluting with an imidazole buffer gradient (500 mM imidazole, 500 mM NaCl, 20 mM Tris HCl). Fractions, including prominent peaks seen on the chromatogram, were tested for the presence of VNNV coat proteinHis6 via Western blot (**Figure 3, B** and **C**).

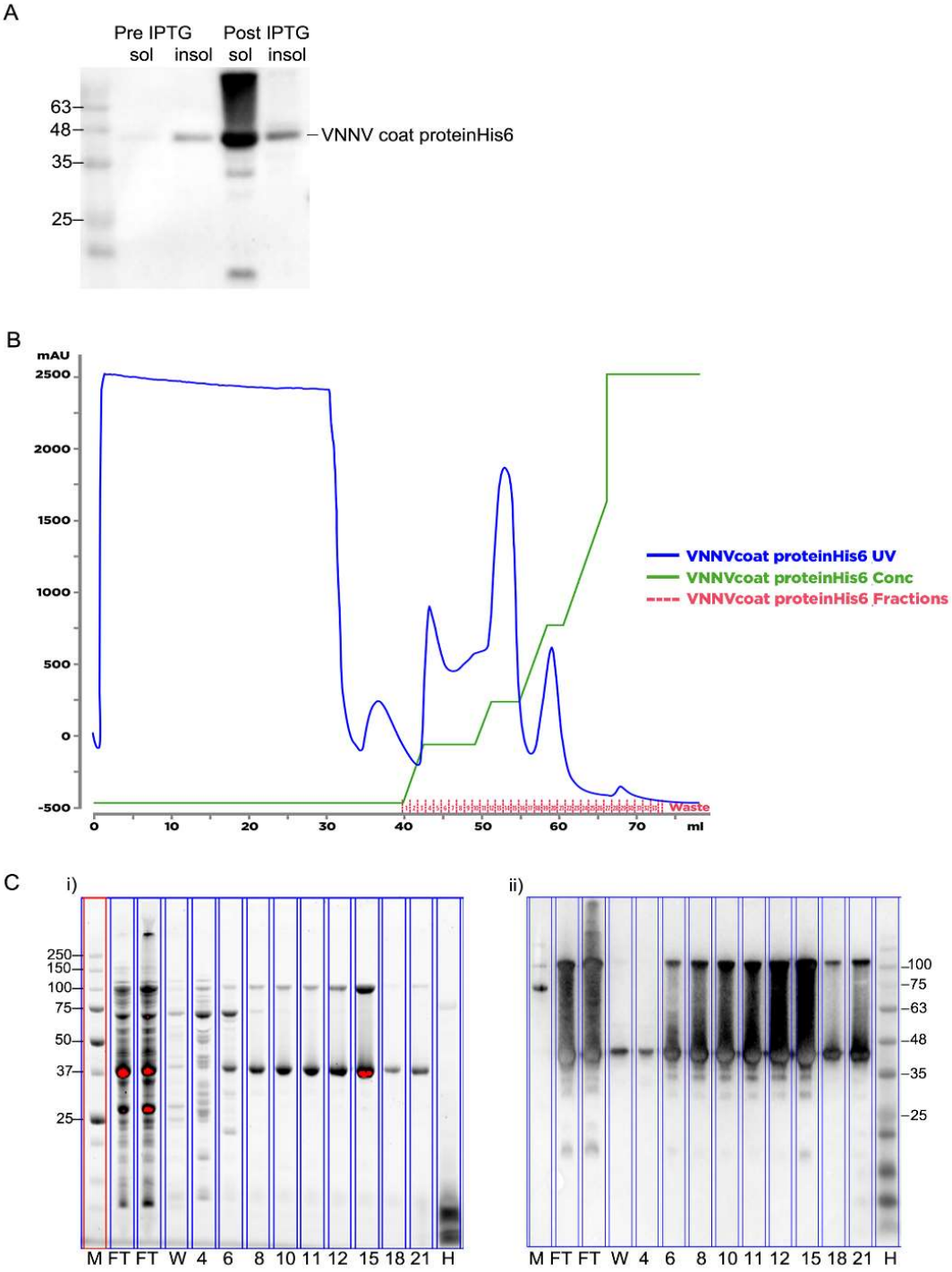

FIGURE 3: Soluble VNNV coat protein production. Using the VNNV-C^{NP} clone, cultures were induced at 37 ºC with 1 mM IPTG & then grown slowly overnight at 16 ºC.

A. Soluble (sol) & insoluble (insol) fractions of VNNV coat proteinHis6 pre IPTG induction & 14 h post IPTG at 16 ºC. Each well on western blot shows protein produced in an equivalent number of cells**.**

B. Chromatogram purification of VNNV coat proteinHis6 by FPLC (fast protein liquid chromatography). Soluble protein purified using 0.1 M $NiCl_2$ on 1 mL HiTrap Chelating HP affinity column, eluting with imidazole gradient. mAU = milli-Absorbance Units. Prominent peaks: fractions 4-6, 10-15 & 18-21.

C. FPLC fractions (B) tested on western blot for presence of VNNV coat proteinHis6 (~38 K Daltons), using **i) total protein detection ii) Anti-His antibody**. M = stain free system total protein marker FT = flow through, W = waste, H = protein marker for western, detecting Histag.

Elutes from the peaks were pooled once the western demonstrated they were all the same protein. The imidazole was dialyzed out at 4 ºC with stirring O/N using a cellulose membrane (Spectra, 6-8 kD MWCO) and three different buffers: 1. Carbonate buffer ($NaCO_3H$ 166 mM) pH 7.4; 2. Carbonate buffer plus salt (NaCl 500 mM) pH 7.4, 3. Tris-HCl 20 mM, 5% Dextrose buffer pH 7.4. Of these, buffer 3, Tris-Dextrose, gave less precipitate O/N than the two other carbonate buffers. The next day the elutes were recovered, traces of precipitate were removed by centrifugation at 15000 x *g* for 30 min at 4 ºC. Soluble protein concentration in the supernatant was determined by a Bradford assay for each of the buffers tested: Buffer 1 = 681.8 ng /µL, Buffer 2 = 704.3 ng /µL and Buffer 3 = 1114.7 ng /µL. Hence buffer 3, Tris Dextrose, was used to dialyse the remaining elute O/N. The soluble protein concentration was determined by a Bradford assay to be 1051.7 ± 85.6 ng /µL. Aliquots were filtered and stored at -80 ºC.

3.3 Immunization via i.p. injection and oral gavage (oral intubation)

Two administration routes were used in parallel: intraperitoneal injection (i.p.) as a reference method to monitor the potential immune stimulus achieved by direct uptake, and oral gavage as a proxy for uptake of the nanoparticle in food. For each administration route 44 fish were vaccinated: 14 with PBS as a control, 14 with 50 µg of VNNV-C^{NP} (dose 1) and 16 with 500 µg of VNNV-C^{NP} (dose 2). For i.p. administration fish were injected with 100 µL of nanoparticle suspension in PBS, or PBS alone using a 25G needle. For oral administration, a novel method was established to administer precise doses. Holding the anaethetized fish lightly around the jaw with the ventral side exposed, a fine, 1.00 mm diameter veterinary cat catheter (Henry Schein Inc.) attached to a 1 mL syringe was inserted down the digestive tract (approx. 35 mm depth) and 80 uL of nanoparticle suspension, or PBS alone was administered. The catheter was withdrawn slowly to avoid regurgitation. The syringe was inverted several times between each administration to ensure the particles were in a homogeneous suspension. Tests were done initially to establish the feasibility of the method and the appropriate administration volume, using a green dye to check if the dose was reaching the intestine. This is shown in **Figure 4.**

Post nanopellet or PBS administration, fish of the same dose from both injection and oral gavage were mixed and redistributed equally into 3 tanks (triplicates), to avoid tank effect. The administration route was easily identified by the colour coding on the fish (**Figure 2**). Tanks were approximately 1 m diameter, containing filtered sea water up to about 30 cm height. Water was flushed frequently, dissolved oxygen ranged from 7.3 to 8.3 mg/L and room temperature from 16.1 to 20.6 ºC, with a natural night/day light regime. There were 9 tanks in total: dose 1, dose 2 and PBS control, all in triplicate. In addition, 12 untreated fish were maintained in a separate tank as sentinels.

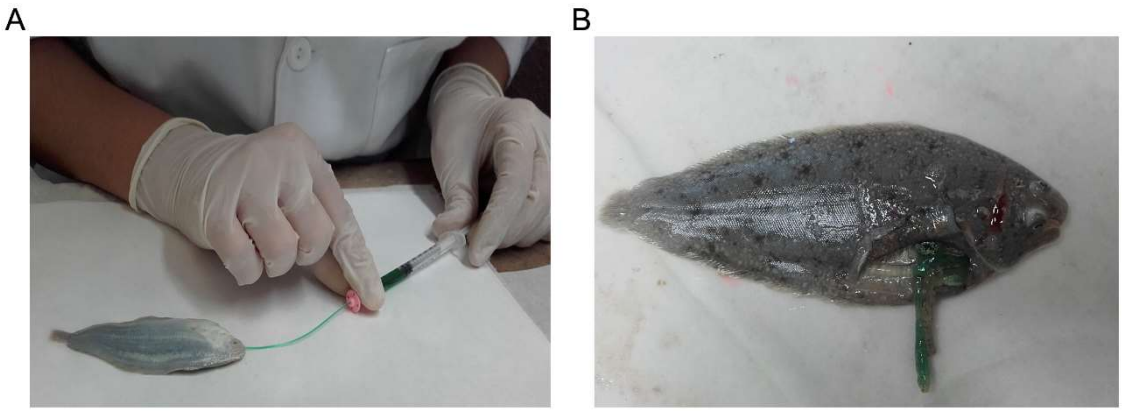

FIGURE 4: Demonstration of oral gavage method to juvenile sole.

A. *S. senegalensis*, approx. 15 g weight, were anaethetized & placed with ventral side exposed. A fine, 1.00 mm diameter veterinary cat catheter, attached to a 1 mL syringe, was inserted down the digestive tract to approximately 35 mm depth. Post administration of liquid, the catheter was withdrawn slowly to avoid regurgitation.

B. Dissected fish showing successful administration to the intestine of up to 80 µL without regurgitation.

On day 13 post administration, fish were not fed. On day 14, a homologous prime boost of nanoparticles at the same dose as initial adminstration was given, delivered in exactly the same way (oral gavage or i.p. injection). Survival and well being were monitored throughout the 6 week experiment.

3.4 Tissue and blood collection

Fish were not fed for 24 h prior to sampling. On day 3 post booster 4 fish were sampled per treatment, namely 1 per dose and adminstration route per tank, plus 1 from any of the triplicate tanks. Fish were weighed (16.6 ± 2.4 g) and blood was taken under anethesia and kept on ice. Then animals were immediately sacrificed and tissue samples were disected out from intestine (~110 mg), spleen (~30 mg) and headkidney (~ 60 mg) (approximate wet weight) and immersed in RNAlater (SIGMA), then stored at -80 ºC. The sex of each animal was also recorded (ratio males:females = 1:3) and animals were checked for signs of any alterations to organs or other malaise. Blood was left to coagulate on ice between 1.5 to 2 h. Samples were then centrifuged at 3000 x *g* for 20 mins at 4 ºC. Sera was recovered and stored at -80 ºC. The remaining fish were maintained in the tanks for a further 4 weeks.

On day 30 post booster, 4 fish per treatment were anaethesized, weighed (26.4 ± 4.3 g) and a blood sample was taken. The fish were euthanized by a quick incision in the brain and the fish was sexed (ratio males : females = 1: 2). All remaining fish were euthanized by anaesthetic overdose. The experimental pipeline, described in sections 3.1, 3.3 and 3.4, is summarised in **Table 1**.

TABLE 1: Experimental pipeline: *S. senegalensis* response to VNNV-C^{NP} vaccination

TIMING	TASKS
Larval stage (pre-mouth opening)	**Fish obtained** *S. senegalensis* larvae were purchased from Cupimar and raised at IFAPA (Instituto de Investigación y Formación Agraria y Pesquera), Cádiz, in an open circulation system of filtered sea water.
Juvenile stage 4 days pre-vaccination	**Colour coding and adaptation.** 100 fish, average weight 15.21 ± 1.93 g, were colour coded by injecting a thin line of dye under the ventral skin: 50 green for injection, 50 red for oral gavage (see Fig 1). Fish were moved to tanks for 3 days adaptation.
DAY 0 vaccination	**Administration of VNNV-C^{NP} nanoparticle by injection i.p. or oral gavage** For each administration route 44 fish were vaccinated: 14 PBS control, 14 dose 1 (50 µg/fish), 16 dose 2 (500 µg/fish). Fish of the same dose from both administration routes were mixed and redistributed equally into 3 tanks (triplicates). 9 tanks in total. 12 untreated fish were kept as sentinels in a separate tank. (See Fig 3 for oral gavage method images)
DAY 14 p.a. post - administration	**Administration of VNNV-C^{NP} booster.** Fish were vaccinated with the same dose of nanoparticle as the initial administration via the same route.
DAY 17 p.a. (3 days post booster)	**Sampling 1** 4 fish per treatment were removed randomly from the tanks to sample. Tissues: intestine, spleen and head kidney; stored in RNAlater at -80ºC Blood: serum was isolated; stored at -80ºC
DAY 45 p.a. (30 days post booster)	**Sampling 2** 4 fish per treatment randomly sampled. Blood: serum was isolated; stored at -80ºC Endpoint.
Throughout experiment	**Outcome: macro-toxicity** Survival and well-being monitored throughout the study period.
Post-experiment	**Outcome: adaptive immune stimulus** Test for anti-VNNV-coat-protein specific IgM in sera from both samplings via ELISA, as a surrogate of protection. Test for gene expression of relevant adaptive immune gene markers via qPCR, using RNA isolated from tissue samples.

3.5 Antibody response (ELISA)

To determine if fish immunized with VNNV-C^{NP} raised specific antibodies against the VNNV coat protein, we performed indirect ELISAs. Serum samples from day 3 post booster (17 days post the first administration) and day 30 post booster were tested from fish immunized via both administration routes and their respective controls. Briefly, Maxisorp 96 microwell plates (Nunc) were coated with 1 µg/well of purified soluble VNNV coat protein (see section 3.2.2) in 50 µL/ well carbonate buffer (100 mM $NaHCO_3$ pH 9.2) O/N at 4 ºC. All further steps were done at RT. Washes between steps were performed in triplicate with TTN buffer (50 mM Tris, 0.05% Tween-20, 0.15 M NaCl pH 7.4). Blocking was done with TTN + 3% BSA (Sigma) for 1.5 h, and serum dilutions were prepared with PBS, 0.05% v/v Tween 20 + 0.5% BSA. Each serum dilution was added in duplicate at 100 µL/well and incubated for 2 h. The primary antibody was a polyclonal anti-Senegalese sole Ig produced commercially in rabbit by Sigma, kindly provided by Dr JJ. Borrego. We used 100 µL/ well of a 1:5000 dilution of the antibody in block buffer, incubating for 1 h. The secondary antibody was an HRP conjugated, γ-chain specific, mouse monoclonal anti-rabbit IgG (Sigma A1949), diluted to 1:4000 with block buffer. We used 100 µL/ well, incubating for 1 h. Detection was via 3,3'5,5' tetramethylbenzine (TMB) substrate reagent set (BD Biosciences): 100 µL/well of TMB freshly prepared in hydrogen peroxide in citric acid buffer pH 3.3 was added and the ELISA plate was incubated in the dark for 20 min at RT. The reaction was quenched by adding 100 µL/well of 2 M H_2SO_4. Absorbance was measured at 450 nm on a spectrophotometer (Victor 3, PerkinElmer). Specific antibody titre was defined as the inverse of the greatest dilution which still gave a positive result. Apart from controls of sera from fish injected or orally administered with PBS, several wells coated with purified *S. senegalensis* Ig were initially included to test the polyclonal antibody, as a positive technical control. In addition, a high total IgM serum sample was included as a comparative reference in some of the plates. This reference serum was a pooled sample extracted from adult Senegalese sole, which had given a strong total IgM signal in previous studies, kindly provided by Dr M. Manchado. Here we used it to demonstrate the specificity of our experiments. Namely, our ELISAs detected antibodies binding specifically to the VNNV coat protein; we were not detecting total IgM. Finally, as a negative control and to determine background signal, several wells were always included in the whole protocol to which no serum was added, only serum dilution buffer.

3.6 RNA extraction and RT-PCR

3.6.1 RNA extraction and cDNA synthesis

RNA was extracted from tissue samples of intestine, spleen and head kidney, taken 3 days post VNNV-C^{NP} booster (see section 3.4). Tissue was removed from RNAlater, blotted and placed

in 200 µl of chilled 1-Thioglycerol Homogenization solution (Promega Maxwell^{R} RSC simply RNA Tissue kit). Tissue was homogenized in a QIAGEN TissueLyser for 2 rounds of 6 min at 50 Hz using 5 mm stainless steel balls and stored on ice for immediate extraction or at -80 ºC long term. Following Promega Maxwell kit instructions, 200 µL of lysis buffer was added, the mixture was vortexed and RNA was extracted in an automated nucleic acid purification system including DNAse treatment (Maxwell RSC Instrument, Promega). RNA was eluted in 45 µL nuclease free water. RNA yield and quality was determined on Nanodrop ND-1000 (Thermo Fisher Scientific) and integrity assessed on the Agilent 2100 Bioanalyzer using RNA 6000 Nano Lab-Chip kit (Agilent Technologies). Then, cDNA was synthesized from 1 µg high quality total RNA using iScript cDNA systhesis kit (Bio-Rad). To test the cDNA, we did conventional PCR using *ef1-α* primers and Biotools PCR kit. PCR products were run on a 1.5% agarose gel.

3.6.2 S. senegalensis primer design and efficiencies

As the full genome sequence of *S. senegalensis* has not been published, primers were designed drawing on sequences in the Andalusian government public database SoleaDB https://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/. After data mining, identities were checked in NCBI gene bank and primers for *cd8-α, cd4, IgM, IgT* and *arg2* were designed using NCBI Primer 3 and Oligoanalyzer 3.1 (Integrated DNA Technologies). The primer sequences and database reference code are listed in **Table 2**.

TABLE 2: *S. senegalensis* primers for qPCR

Gene	Sequence 5'-3'	Amplicon Size (bp)	Reference/ Accession #/ SoleaDB
ef1-α	**F**: GATTGACCGTCGTTCTGGCAAGAAGC **R**: GGCAAAGCGACCAAGGGGAGCAT	142	Infante, 2008 Genebank: AB326302
cd8-α	**F:** GTCGCAGTTCTGCTCTCCGC **R:** TCGGTTGCAGTAGAGGACGG	97	solea_v4.1_unigene59609
cd4	**F:** AGCAGGGCAGAGAAGAAGACG **R:** GCAGCTGGCCGGGATGTAAG	142	solea_v4.1_unigene450963
IgM	**F:** TGAAACATTGACACAGCCAGCC **R:** CGTGTGAGCTTCCAATCCACTC	149	solea_v4.1_unigene691100
IgT	**F:** AGTGGTAAAGCGGCCTGGAG **R:** GCCTTTCCTTCAGCTTGTCTG	108	solea_v4.1_unigene625671
arg2	**F:** ACCGCGTCGTTAGCAGTTGA **R:** TGCTCTGTGTCGTCCTTCGCC	107	solea_v4.1_unigene32066

A published primer was used for the reference gene *ef1-α* (25). Primer efficiencies were determined by qPCR, performed at 60°C annealing temperature using iTaq Universal SYBR Green Supermix (Bio-Rad) as follows: 1) 95.0ºC for 3 mins (hot start), 2) 95.0ºC for 10 sec, 3) 60ºC for 30 sec. Plate read. 4) Go to 2, 39 repeats (40 cycles total), 5) Melt curve 65.0ºC to 95.0ºC. Plate read. We ran serial dilutions of control cDNA in triplicate for all tissues for *ef1-α* and from head kidney and spleen for the other genes, as intestine cDNA was scarce. Efficiency E= $10^{-1/slope}$ and % Efficiency= (E-1) x 100% was calculated according to BioRad Real-time PCR quick guide (2006) pag. 4-6, where 2^n = dilution factor. Namely, n = $\log_2$(dilution factor) was plotted on the x axis with the Cq values on the y axis (26), then linear regression was performed with Prism 6.01 (GraphPad). **Figure 5** shows the graphs and percentage efficiencies for the 6 genes tested.

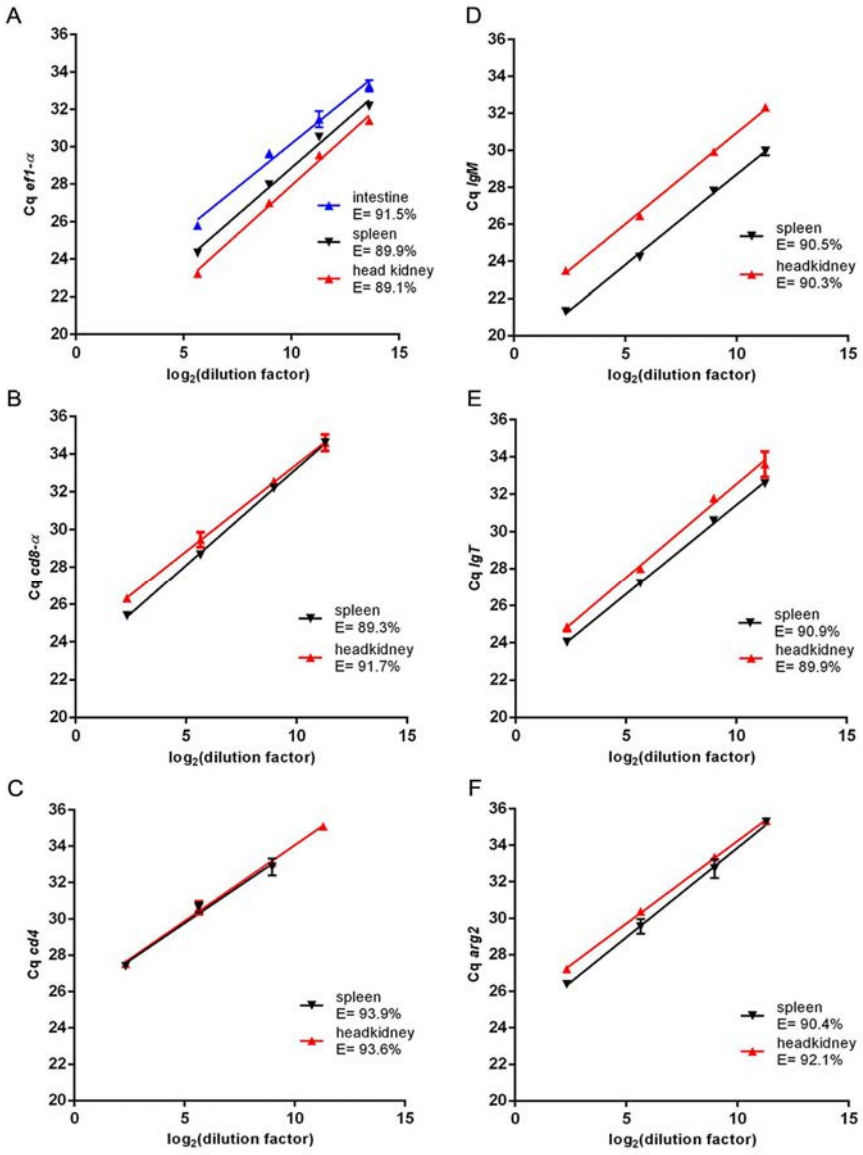

FIGURE 5: Efficiencies of *S. senegalensis* primers for qPCR.

Primer efficiencies determined via qPCR using serial dilutions in triplicate of control cDNA. Efficiencies (E= $10^{-1/slope}$) calculated via linear regression for primers: **A.** *ef1-α* **B**. *cd8-α* **C**. *cd4* **D**. *IgM* **E**. *IgT* **F**. *arg*

There were 3 other primers which we tried using, *il-10*, *mx* and *ifn 1*. All were at the limit of detection in qPCR as they gave low basal expression in control fish (Cq values ~ 35), especially in intestine. The results, which were erratic or showed negligible changes, were not considered reliable. The *ifn 1* primer was also tested over a temperature gradient to try and improve performance. But finally, we did not pursue further experiments with these 3 primers.

3.6.3 RT-PCR

From primer efficiency tests we were also able to determine what was the appropriate dilution of cDNA to achieve a Cq for the reference gene within ± 3 cycles in relation to the Cqs for the genes of interest using control cDNA. We aimed for Cqs of in the range 20 - 25 and established two groups: For primers *IgM, cd8-α* and *IgT*, we used cDNA at 1:50 dilution for *ef1-α* and for gene of interest at 1:5. For primers *arg 2* and *cd4* we used cDNA of *ef1-α* at 1:200 and gene of interest at 1:5. Quantitative real-time PCR (qPCR) was performed at 60 °C annealing temperature using iTaq Universal SYBR Green Supermix (Bio-Rad) with 250 nM of primers and 2.5 µl of cDNA previously diluted to 1:5 for the target and 1:50 or 1:200 for the reference gene, *ef1-α*, as explained above. Reaction conditions were as given in section 3.6.2. All the samples (N = 4 per treatment) were run in triplicate, and data were analyzed for individual replicates using the Livak method (27). Statistical analysis used a one-way unpaired t-test to compare each gene's mean fold change in expression with control, using Welch's correction for unequal variances (Prism 6.01, GraphPad) and with $p < 0.05$ considered statistically significant.

3.7 Ethics statement

The experiments with *S. senegalensis* juveniles were performed at IFAPA (Instituto de Investigación y Formación Agraria y Pesquera) in the center "El Toruño", Puerto de Santa Maria, Cadiz; under the guidance and supervision of Dr M. Manchado. All methods were performed in accordance with the Spanish Royal Decree RD 53/2013 and EU Directive 2010/63/EU for the protection of animals used for research experimentation and other scientific purposes.

4. Results

4.1 Fish survival and well-being

During the 7 week experimental period, fish did not show any signs of malaise post nanoparticle administration. There was 1 death in the PBS control group the first 24 h after the initial i.p.

injection, which we attribute to the injection procedure. There were no deaths of orally intubated fish. When blood and tissue samples were taken 3 days post booster (day 17 post initial administration) and at the endpoint, the animals were checked for abnormalities. No signs of morphological alterations were observed such as softening of liver or enlarged spleen. The eyes of the fish were bright and skin healthy.

4.2 Specific antibody response (ELISAs)

Fish immunized with VNNV-C^{NP} via both routes raised specific antibodies against the VNNV coat protein to different degrees. As we have measured serum antibodies, we consider the response detected is predominantly IgM, although we used a polyclonal antibody, as IgM is the prevailing isotype in fish sera. For example, in rainbow trout (*Oncorhynchus mykiss*) the concentration of IgM in serum (2,520 µg/mL) is far higher than IgT (3.7 µg/mL) (28). Thus, we refer to the antibodies detected here as anti-VNNV coat protein IgMs. **Figure 6** shows the antibody titration curves for injected fish and orally intubated fish **Figure 6A** and **B**, at 3 and 30 days post booster (dpb), **Figure 6 i** and **ii**. In injected fish, specific anti-VNNV coat protein IgMs were already clearly present by day 17 post initial administration (3 dpb). Both low and high doses had evoked a similar amount of specific antibody with titre 2500. By 30 dpb substantial antibody expansion had taken place and was more pronounced in the high dose group. The titre for both high and low dose was ≥ 12500. Note **Figure 6A ii** includes a pooled adult serum sample known to have high total IgM. The titration curve shows this reference sample contains some antibodies which can recognize the VNNV coat protein compared to untreated control, but the levels are far less than that found in VNNV-C^{NP} vaccinated juveniles, validating we are detecting specific rather than total IgM.

In orally administrated fish, the specific antibody response was present but low. At 3 dpb, error bars overlap between the control and low dose group, hence the response is not clear. The high dose group has significantly more antibodies than control, titer 300. At 30 dpb the pattern has changed. Some antibody expansion was detected in the low dose group (50 µg/ fish), but in the high dose group (500 µg/ fish) there appears to be antibody suppression. To visualize this better, absorbance values for all the treatment groups at 1:100 serum dilution were plotted, and statistical analysis performed.

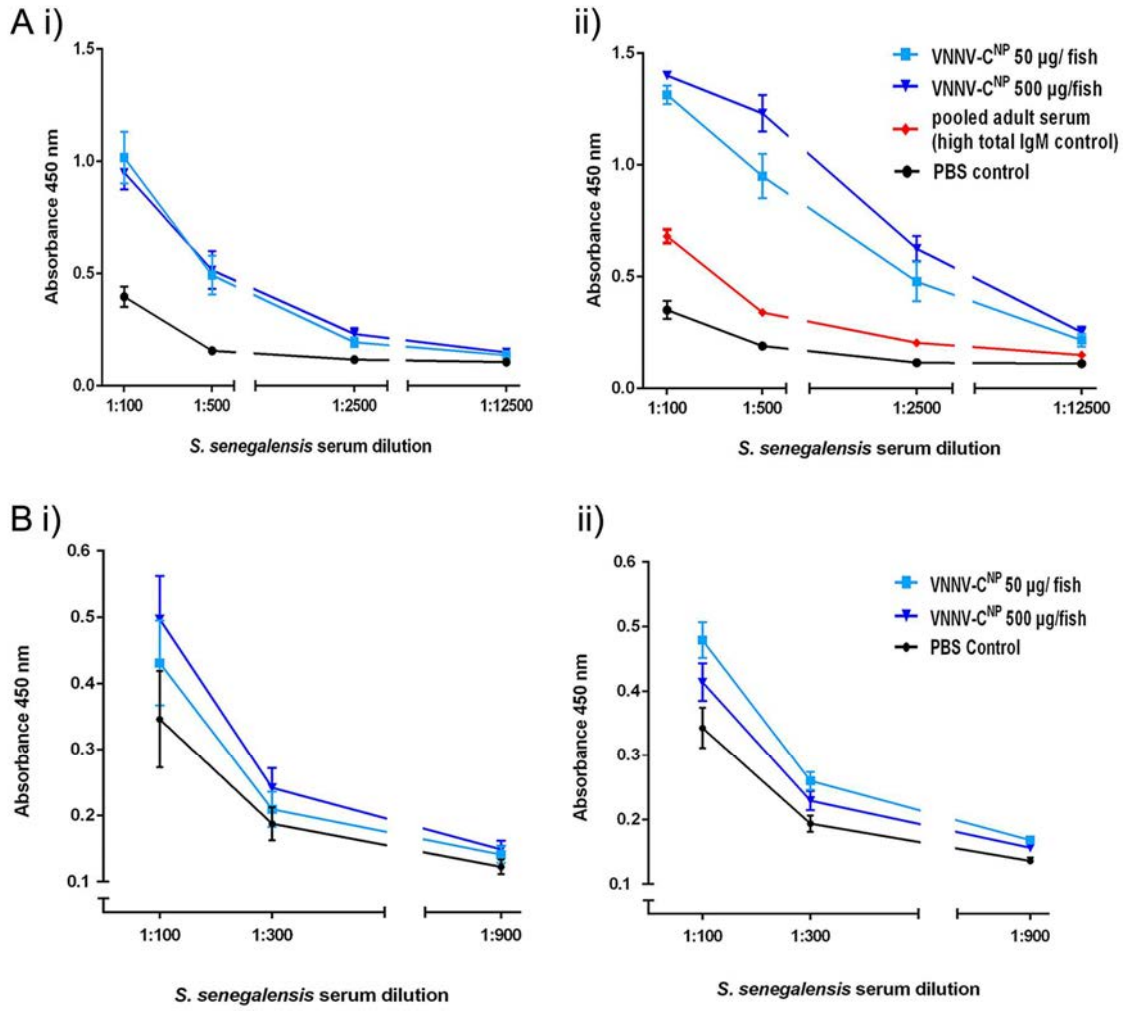

FIGURE 6: Titration curves for Anti-VNNV coat protein IgM in *S. senegalensis* sera from juveniles vaccinated with VNNV-C^{NP}, determined by ELISA. Fish (~15g) were administered 50 µg (■) or 500 µg (▼) of nanoparticle in PBS on days 0 & 14 (booster) by **A. i.p. injection** or **B. oral gavage**. Blood was sampled (n=4) at **i) 3 days post booster** & **ii) 30 days post booster**. Controls were PBS (●) administered same routes & times, & pooled adult serum high in total IgM (♦) as reference serum. Data are mean ± SD.

Figure 7 shows clearly the greater response evoked by injecting the nanoparticle i.p. compared to oral gavage. Also, the statistically significant antibody expansion from 3 to 30 dpb in the injected fish is evident. In orally intubated fish, the systemic IgM response is much less. At 3 dpb only fish intubated with the high dose have a significant level of antibodies compared to control. However, by 30 dpb, in fish treated with the low dose the level has increased significantly, while in those treated with high dose the level was slightly reduced.

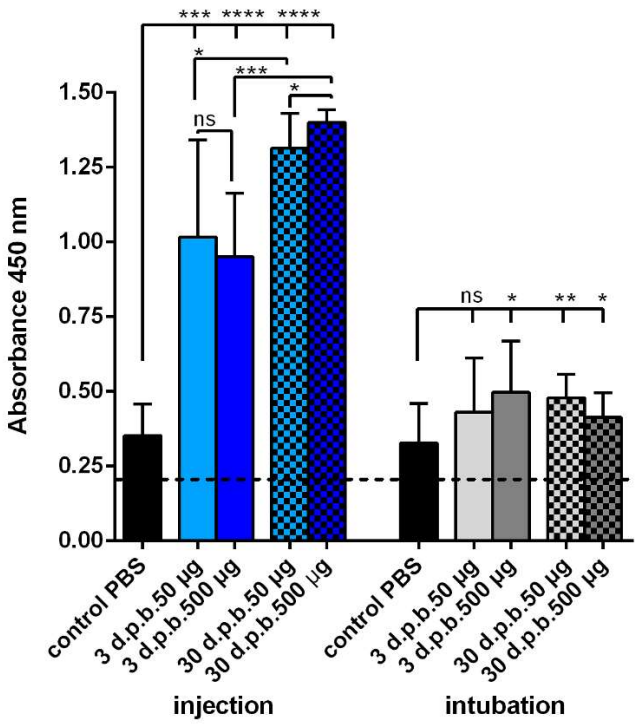

FIGURE 7: Comparison of anti-VNNV-coat protein IgM in *S. senegalensis* sera at a 1:100 dilution from juveniles vaccinated with VNNV-C^{NP}. Fish (~15 g) were administered 50 µg or 500 µg of nanopellet (NP) in PBS on days 0 and 14 (booster) by i.p. injection (left) or oral gavage (intubation) (right).

Anti-VNNV-coat protein IgM was determined by ELISA at days 3 and 30 post booster (n=4). Control was sera from fish administered PBS by same routes & times. Broken horizontal line is the background cut off (2 x absorbance without serum). Data are mean ± SD. Differences between each treatment mean & control, & between treatments of the same administration route were analyzed by unpaired one-sided t-tests with Welch's correction for unequal variances. Significance levels *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, ns = not significant.

4.3 RNA quality

All head kidney and spleen samples gave good yields (approximately 500-1200 ng/µL in a total volume of 45 µL) and the spectrophotometer ratios 260/280 and 260/230 were between 2.00 and 2.20, indicative of high purity. Intestine samples varied in yield and quality. Some were similar to the excellent head kidney and spleen results. Others were of medium quantity and quality e.g. 220 ng/µL with spectrophotometer ratios 1.93 and 1.36. These samples needed to be tested on the Bioanalyzer. A few intestine samples were clearly degraded e.g. 101 ng/µL with ratios 1.4 and 0.51. These were discarded. Bioanalyzer results showed RNA integrity number (RIN) values for the general samples were excellent from 9.1-10. Of the medium quality intestine samples, all had good RINs of 8.5- 9.5 and 1 was 7.9. A degraded sample gave a negative result as expected.

4.4 Gene Expression (qPCR)

To try to gain more insight into the sero-conversion process, we looked at the expression of immune-related genes at 3 dpb (17 days post first administration). **Figure 8** shows the fold change in gene expression for fish administrated 500 µg of VNNV-C^{NP} via injection (A) and oral gavage (B) for all genes and tissues tested.

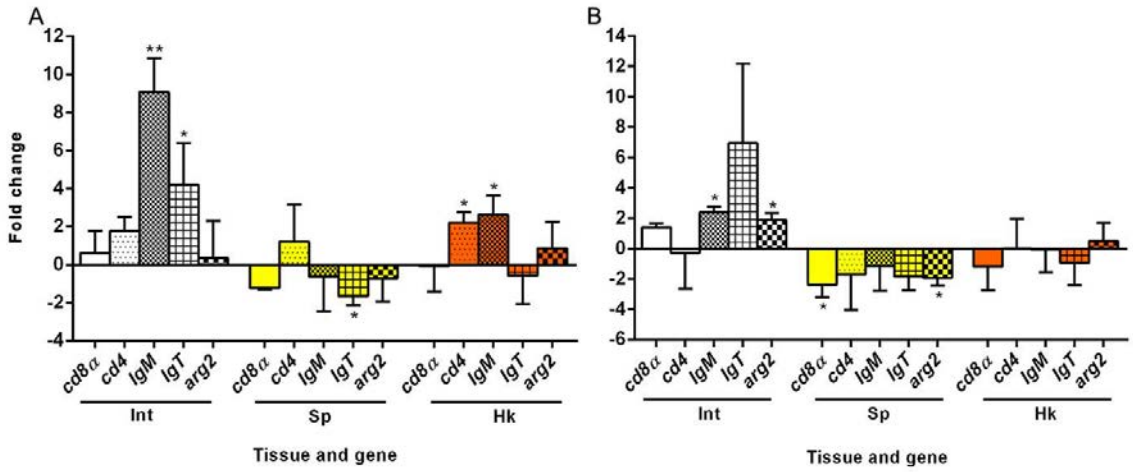

FIGURE 8: Gene expression stimulated by VNNV-C^{NP} comparing 5 genes grouped by tissue for A. Injection & B. Oral gavage. Fish (~15 g) were administered 500 µg VNNV-C^{NP} nanopellet (NP) in PBS on days 0 and 14 (booster) by A. i.p. injection or B. oral gavage (intubation). Tissues sampled from 4 fish/ treatment for RNA extraction 3 days post booster: Int = intestine, Sp = spleen, Hk = headkidney. Gene expression determined by qPCR with *ef1-α* as reference gene. Data are mean ± SD (n = 4). Differences between each treatment mean & control analysed by unpaired one-sided t-tests with Welch's correction for unequal variances. Significance levels *p < 0.05; **p < 0.01

At this high dose *IgM* and *IgT* were upregulated in the intestine via both administration routes, though *IgM* was particularly high in injected fish, 9.07 fold compared to 2.39 fold in orally intubated fish, while *IgT* was upregulated in both, and more prominently in intubated fish, 6.96 fold compared to 4.21 fold in injected fish. *Arg2*, which is a putative indicator of alternatively activated macrophages in fish (29) was also significantly but slightly upregulated in the intestine of the intubated group; 1.88 fold. In the spleen there was a slight tendency for down regulation in all genes tested in the intubated fish. In the head kidney, where induction of gene expression is an indicator of a systemic response (30), *cd4* and *IgM* were upregulated in the injected fish, 2.21 and 2.64 fold respectively. In intubated fish there were no significant changes in head kidney. Note that *cd8* was not significantly upregulated in any of the scenarios. This could indicate a CD8+ T cell response is not being activated, though we see *cd4* upregulated and a B cell response (antibodies). This situation has been reported in response to certain other recombinant protein antigens (31) and is considered a common handicap of non-replicative vaccines, which then may require adjuvants (32). The only significant response for *cd8* was down regulation (-2.38 fold) in the spleen of orally intubated fish. In addition, we tried looking at the expression of *il-10* as a possible indicator of a tolerance response, but gene expression was very low or negligible in all samples and at the limits of reliable detection (data not shown). It may be necessary to use purified cell populations instead of whole tissue samples to be able to measure this cytokine and to link results meaningfully to cell types (33).

We then compared the expression of *IgM*, *IgT* and *arg2* in fish treated with low and high doses, of VNNV-C^{NP}, in all the tissues sampled. **Figure 9** shows the gene expression results according to tissue, administration route, gene and nanoparticle dose.

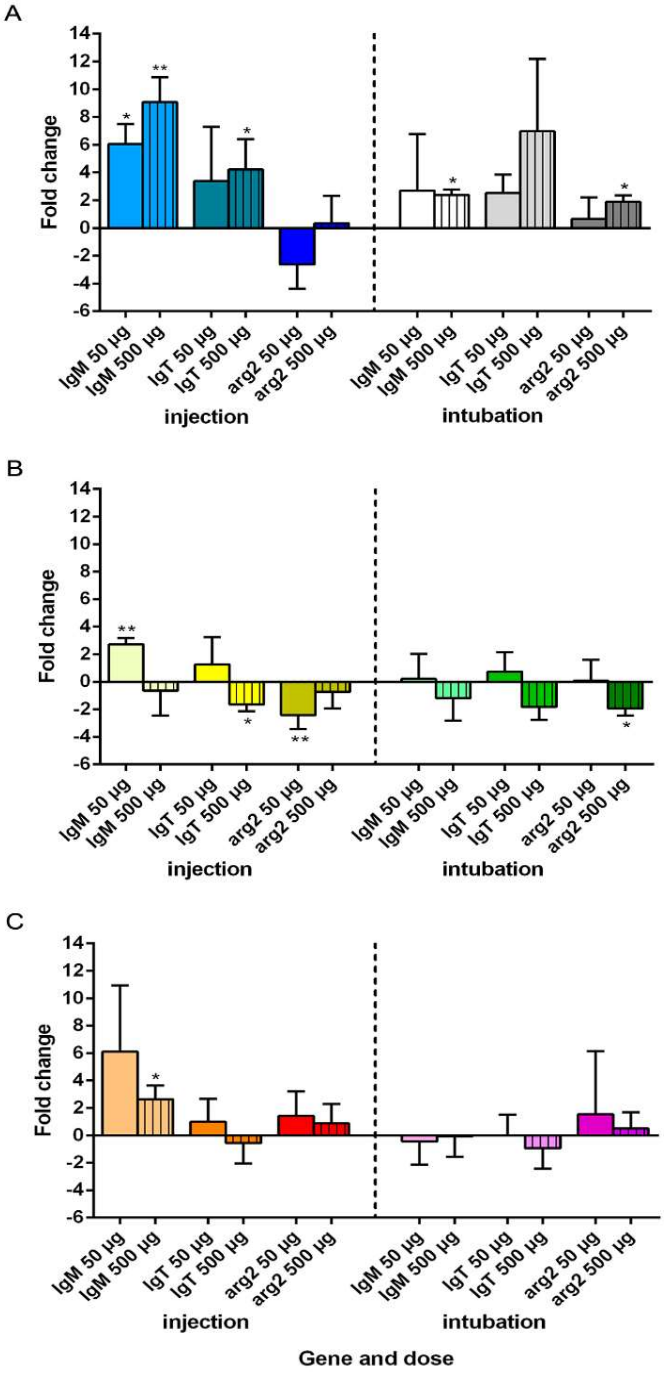

FIGURE 9: Gene expression via qPCR comparing *IgM*, *IgT* and *arg2* for high & low dose of VNNV-C^{NP} grouped by administration route in tissues:

A. Intestine B. Spleen C. Head kidney.

Fish (~15 g) were administered 50 or 500 µg of VNNV-C^{NP} in PBS on days 0 and 14 (booster) by i.p. injection (left) or oral gavage (intubation) (right).

At 3 days post booster tissues were sampled from 4 fish/ treatment for RNA extraction. Gene expression determined by qPCR with *ef1-α* as reference gene. Data are mean ± SD (n = 4). Differences between each treatment mean & control were analysed by unpaired one-sided t-tests with Welch's correction for unequal variances. Significance levels *p < 0.05; **p < 0.01

Results of most interest are: in **intestine** (**Figure 9A**) there is a dose related upregulation of *IgM* (6.04 and 9.07) and *IgT* (3.39 and 4.21) in injected fish, and of *IgT* (2.52 and 6.96) in orally intubated fish, for low and high dose respectively. While we expected upregulation of *IgT* in the

intestine, especially in intubated fish, *IgM* upregulation in the intestine post i.p. injection came as a surprise to us, although a considerable population of IgM^+ B cells have been reported in the intestine of rainbow trout (28). The other point of interest is *arg2* was downregulated (-2.64), though not significantly, in fish injected with a low dose of nanoparticle and upregulated (1.88) in fish intubated with a high dose of nanoparticle. This is evaluated in the discussion section. In **spleen** (**Figure 9B**) *IgM* was upregulated (2.71) and *arg2* was downregulated (-2.41) in fish injected with a low dose of nanoparticle. For intubated fish the only significant change in spleen was *arg2* was downregulated (-1.94) with a high dose of nanoparticle. In **head kidney** (**Figure 9C**) *IgM* was upregulated in injected fish, 6.10 and 2.64 fold for low and high dose respectively, though the low dose result has a very large SD and hence is not significant.

5. Discussion

The fundamental result from this work is that VNNV-C^{NP} can evoke the production of specific antibodies against the RGNNV/SJNNV viral capsid protein *in vivo*. This is a key surrogate of protection (24) and it has been achieved in a primary target group for vaccination: juvenile fish of a susceptible farmed species. Further, there was a considerable antibody expansion after i.p. homologous prime boost, indicating immune memory had been triggered. The immunogenic potential of this protein nanoparticle in *S. senegalensis* is therefore substantial and was evidenced without adding any adjuvant.

The question is then how to exploit this potential. Intraperitoneal injection raised far more antibodies than oral administration and stimulated *cd4* and *IgM* in the head kidney (**Figure 8A**), indicative of an adaptive systemic response. Knowing that specific antibodies can be raised, the next step would be to see if i.p. administration can provide protection in a challenge model, which had not been logistically possible at the time of these experiments. With a challenge model a correlate of protection could be established, relating dose and percentage survival statistically (34). This is an essential step in verifying the protective potential of the nanoparticle. But, once protective capacity is established, practical considerations need to be addressed. The high cost and impracticality of vaccinating larvae and juveniles i.p. may veto administering by injection in fish farms, except to broodstock.

It is therefore highly desirable to explore other routes, particularly oral administration, which is considered the most widely applicable for fish of any size, is easy for farmers and less stressful for fish (35). Along these lines, the first contribution this paper makes is to set up an oral gavage or intubation method for sole. Oral gavage methods have been published for zebrafish (36) and trout (37), and are applicable for testing many compounds such as toxins and immunostimulants,

as well as oral vaccine candidates. As far as we are aware, this is the first time such a method has been reported for sole. It was cheap and easy to set up, not difficult to perform and fish showed no subsequent signs of malaise. Success of the method is shown by the gene expression results for intestine (**Figure 9A**, right), since *IgT* was upregulated in a dose dependent fashion in intubated fish. Also, systemic antibodies were raised to a different extent depending on oral dose (**Figure 7**). The method therefore can deliver precise doses to the gut without injuring the fish.

Concerning the raising of antibodies and gene expression in orally intubated fish, we see a dose dependent mucosal immune response: *IgT* was upregulated (**Figure 9A**, right). However, the systemic antibody response is low (**Figures 6B** and **7**), especially when considering the potential immunogenicity demonstrated in the injected group. In addition, using oral gavage with the high dose, antibodies decreased rather than increased after the homologous booster (**Figure 7**). This is a sign of antibody suppression or deletion (anergy) which is a form of tolerance (hypo responsiveness) reported to occur with high doses of orally delivered antigen in mammals (38). In salmon, a reduction in serum antibodies was reported after two oral administrations 7 weeks apart using alginate encapsulated inactivated infectious pancreatic necrosis virus (IPNV) (30). Whereas other authors showed antibodies increased with time when an oral vaccine was delivered 3 days/week for two months. But the same vaccine induced tolerance when given 5 days per month (39). The tuning of dose and administration regime is therefore critical to achieve a systemic response. Indeed, regulatory T-cell (Treg) induction is the form of tolerance associated with low antigen doses and tolerance is the "default immune pathway" in the intestine (38). In our case, the low dose was more favourable than the high, but still it only weakly induced antibody production. It is unfortunate we were not able to measure expression of *il-10* as a mediator of immunosuppression (40), since *il-10* was at the limit of detection in qPCR as explained in section 3.6.2.

Instead, we included *arg2* which is one of the two isoforms of arginase found in vertebrates. Arginase is known to supress iNOS activity and induction of the inflammatory response because both arginase and iNOS compete for a common substrate L-arginine (33). Therefore, upregulating *arg2* may lead to an anti-inflammatory or "healing response" characterized by M2 macrophages (29). We used this gene as a proxy for an anti-inflammatory environment (29). We found *arg2* was upregulated, slightly, in the intestines of fish intubated with the high dose. In general, however, the results obtained using this gene marker in our context (total tissue RNA and not purified cell populations), were minor and did not provide a clear pattern. The antibody response, however, indicated that tolerance was likely to be occurring and both anergy and Treg induction mechanisms could be involved. In future work it could be useful to check gene expression of *tgfβ* or *foxP3* as indicators of Treg differentiation (35) if sequences are available for *S. senegalensis*.

Note another limitation of the gene expression results is that tissue sampling was done at only 1 time point; 3 days post booster. So, we only have a snapshot of gene expression at that moment.

In order to optimise a response via the oral route there are various points to consider. First, to achieve a systemic response the nanoparticle needs to be sufficiently robust to resist the high pH and enzymatic degradation in the gut and be taken up and reach the immune related cells in the lamina propria. The lamina propria along with intraepithelial lymphocyte compartments (IEL) are the main effector sites in the teleost gut-associated lymphoid tissue (GALT) (41). With flow cytometry and analysis of tissue sections, our group has previously demonstrated that our nanostructured proteins are taken up by intestinal cells and reach the lamina propria, using a fluorescently labelled TNFα inclusion body orally administered to trout (42) and zebrafish (36). We have also demonstrated the nanoparticle used in the present study, VNNV-C^{NP}, is taken up by zebrafish intestinal cells when orally intubated (23). The average size of the particle, 422 ± 87 nm measured by field emission scanning electron microscopy (23), is similar to the size range of antigen loaded PLGA nanoparticles (450-500 nm) which induced protective immunity against VHSV in olive flounder juveniles (43).

This leads us to a second critical consideration, the administration route. While protection elicited by oral vaccines alone has been variable (35), oral administration as a heterologous booster can be effective. Heterologous boosting is known to raise a stronger response than homologous boosting since it is more likely to induce both humoral and cell-mediated immune (CMI) responses (31, 32). In their study in olive flounder using the viral haemorrhagic septicaemia virus (VHSV), Kole et al., 2019, used 2 administration scenarios to flatfish of very similar size to our Senegalese sole: a primary immersion immunization with inactivated-VHSV-loaded PLGA nanoparticles, followed two weeks later by either 1) a homologous booster by immersion or 2) a heterologous oral booster, introducing the loaded nanoparticle into the feed for 2 consecutive days. Results in terms of relative percentage survival (RPS) after VHSV challenge were higher for the immersion/oral group than immersion/ immersion, 73.3% versus 60% respectively. And, in a pilot study with an oral/oral administration strategy, only 23.3% RPS was obtained (43). This exemplifies the need to try different modes and combinations of delivery to optimize the response.

An advantage of immersion is that it exposes fish to infection by the natural portals of pathogen entry for horizontal transmission. Betanodavirus infection has been postulated to occur via the nasal cavity in ~1 year-old sevenband grouper (44). The virus penetrates the nasal epithelium, then passes thorough the olfactory nerve and bulb to the olfactory lobe, where most viral replication was observed. However in striped jack larvae, initial viral multiplication was reported in the spinal chord (45), and lesions in the brain and retina occurred later in infection. As primary

sites of lesions within the nervous system differ between fish species, there does not appear to be a single route of infection and the pathways of entry remain unclear (1).

Regarding oral vaccination against VER, two recent publications have shown very promising results using VLPs. In convict grouper (*Epinephelus septemfasciatus*), Wi et al, 2015, have demonstrated 100% survival with i.p. injection of RGNNV VLPs produced in yeast (*Saccharomyces cerevisiae*) against an RGNNV challenge and 57% survival when the VLPs were administered by oral gavage, without adjuvant, in a single dose of 50 µg to fish weighing 71.5 g (19). In orange spotted grouper (*Epinephelus coioides*), Chien et al, 2018, tested 3 different administration routes, oral in the feed, immersion and injection i.m. of OSGNNV (RGNNV) VLPs produced in *E. coli* and then cell-free self-assembled (18). Very interestingly, Chien et al. used grouper larvae and optimized the oral dosage regime in terms of quantity and number of repeated doses to achieve the best RPS after challenge. Results showed immersion gave the highest RPS, 81.9, then injection, 61.4, and oral, 52.3, feeding the larvae 4 times with a diet supplemented with 200 µg/g VLPs with a 7 day interval between each administration. Note while in both of these studies the oral route did not give the highest protection, the authors consider it to be very encouraging, stressing again the overriding practicality and cost effectiveness of oral vaccination. It should be mentioned that immersion is also a feasible method for larvae and very young fish, though it requires using substantially more vaccine than via the oral or injection route. Immersion mimics a natural waterborne infection of the virus and in this sense may optimize immune induction (35). This is corroborated by Chien et al's results in grouper larvae.

Concerning advantages of our nanoparticles over VLPs, the greatest difference is in the ease and low production cost of our biomaterial, which is purified by enzymatic and mechanical disruption (42). This is a far more straightforward and less costly approach than required to produce VLPs (46). In addition, our nanoparticles can be lyophilized, retaining biological activity (42). Therefore, transport and storage would not require the cold chain. Another attractive feature we have already mentioned is that impurities from the production process are immunostimulants for fish, namely LPS, peptidoglycan and nucleic acids. However, given the present results, we could also consider testing an additional adjuvant for the oral route to enhance antigen uptake and presentation (32).

In summary, there are many avenues to pursue to enhance and optimize the response. The critical next steps are to show protection through challenge models, to explore the immersion route, try heterologous oral boosting as well as optimize the oral dose regime and timing. The immunogenicity of VNNV-C^{NP} when injected i.p. to sole juveniles is very clear with the production and expansion of specific anti-VNNV capsid antibodies. The protein nanoparticles are innocuous to the fish, should not accumulate in the environment and bulk production is cheap and

feasible. We are therefore very interested to continue development of this nanoparticle as a potential vaccine candidate.

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CHAPTER 3

IN VIVO STUDIES IN FARMED FISH MODELS (2): VHSV-G-frg16^{NP} and rainbow trout *(Oncorhynchus mykiss)*

A VHSV-G protein fragment, nanostructured as inclusion bodies, raises specific, functional antiviral antibodies in trout, demonstrating its potential as a fish vaccine candidate.

1. Abstract

Developing effective, practical vaccines against viral diseases in farmed fish is a priority for the aquaculture industry. We have drawn on recent work in biomaterials science to seek innovative strategies. Bacterial inclusion bodies (IBs) have been shown to be biologically active, providing a slow release of native and native-like proteins from a protective amyloid-like scaffold. We have embraced this concept and nanostructured fish viral proteins as IBs. The attractiveness of IBs as a vaccine design for aquaculture is that they are safe and stable *in vivo* without encapsulation, in contrast to soluble proteins. They are cheap and easy to produce, are fully scalable and carry bacterial remnants such as lipopolysaccharide and nucleic acids, which are demonstrated immunostimulants for fish. We have produced protein nanoparticles as IBs made of the C-terminal half of the viral haemorrhagic septicaemia virus (VHSV) G protein. Here we test their immunogenicity in a trout (*Oncorhynchus mykiss*) infection model using fingerling fish. Results from ELISA show specific anti-VHSV IgM antibodies were raised by 30 days post i.p injection of the nanoparticles. Among these, we detected the presence of neutralizing antibodies by a viral neutralization assay in fathead minnow *epithelioma papulosum cyprini* (EPC) carp cell line. Post VHSV infection, the specific anti-VHSV IgM antibody titre expanded significantly in the vaccinated group, compared to sham-vaccinated fish. We thus show viral proteins nanostructured as IBs can evoke specific, functional anti-viral antibodies in fish, demonstrating the potential of this strategy for vaccine development.

2. Introduction

Vaccine development against viral diseases in farmed fish is a prime concern in aquaculture. The number of commercially available vaccines is low considering the size and diversity of the industry (1) and the emergence of viral diseases has increased through intensive rearing and rapid global expansion (2). There is emphasis on finding practical solutions so that fish, particularly juveniles, can be mass vaccinated in a cost-effective manner. In this context, we have drawn on recent work in biomaterials science for alternative strategies. Bacterial inclusion bodies (IBs) have gained interest as tuneable, functional, non-toxic protein nanoparticles (3). They are biologically active, with a high propensity to cross cell membranes and can function as protein releasing agents (4). Embracing this concept, we have nanostructured fish viral proteins as IBs in an innovative vaccine strategy. The advantages of this approach for fish vaccines are many. IBs are stable *in vivo* without encapsulation and are cheap and easy to produce in *Escherichia coli* (5). They could be injected or mixed in the feed and can retain functionality across a wide pH range and after lyophilization (6). In addition, they carry bacterial remnants as such as lipopolysaccharide, peptidoglycans and nucleic acids as impurities, which are immunostimulants for fish (7).

We have already produced IBs from two antigenic proteins of viruses affecting salmonids. We demonstrated the uptake of these nanoparticles *in vitro* in zebrafish liver (ZFL) cell line and *in vivo* by zebrafish. We also showed the viral protein IBs elicit an innate anti-viral immune response in rainbow trout head kidney macrophages (8). Here we focus on one of these nanoparticles, VHSV-G-frg16^{NP}, made of a fragment of the glycoprotein (G) of viral haemorrhagic septicaemia virus (VHSV). VHSV is a Novirhabdovirus with a negative-stranded RNA linear genome of approximately 11 kb, whose organization is shown in **Figure 1A** (9), with related structural components in **Figure 1B**. The drawing shows the bullet shaped virus, ~170-180 nm in length, with the G protein protruding from the envelope as a trimeric spike. The G protein of rhabdoviruses facilitates viral attachment and entry to the host cell, thought to be initiated through protein receptor interactions (10).

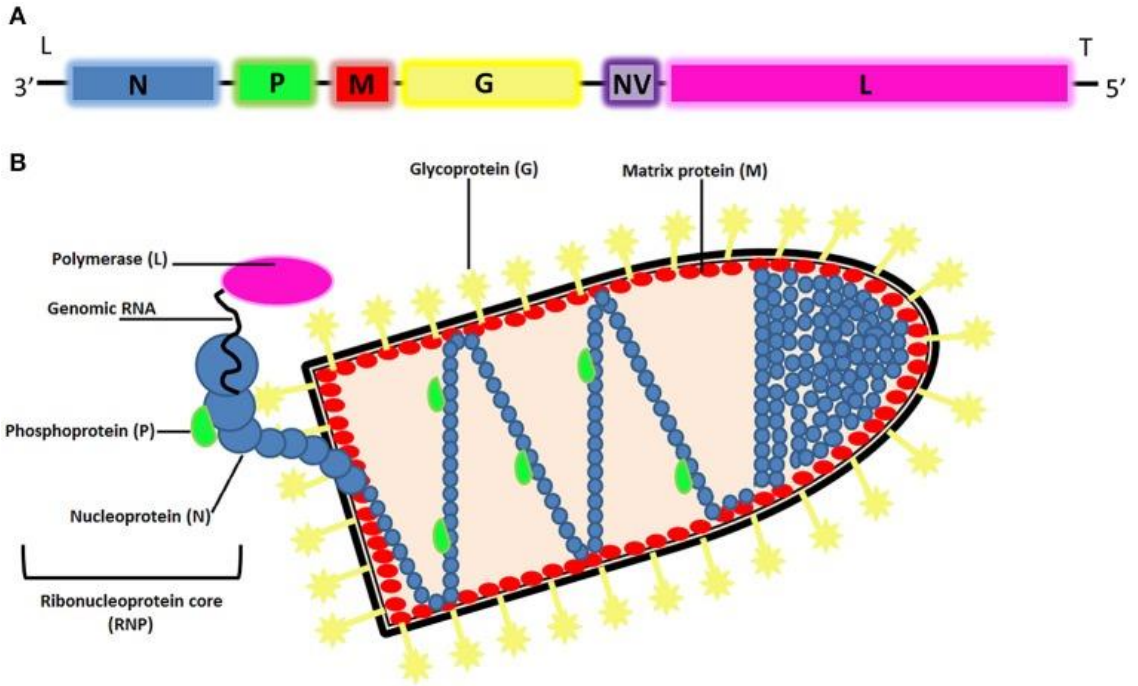


FIGURE 1: Schema of the VHSV genome and rhabdovirus features

A. The -ssRNA genome of VHSV with gene order 3'-leader-N-P-M-G-NV-L-trailer-5'. **B.** Morphology & structural components. Reprinted with permission from (9).

Viral haemorrhagic septicaemia (VHS) continues to be listed by the World Organization for Animal Health (OIE) as a notifiable fish disease, demonstrating its global impact and the need for containment http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2019/ . The rhabdovirus VHSV causes outbreaks in both freshwater and marine fish including farmed rainbow trout (11), turbot, reviewed in (9), olive flounder (12) and wild fish such as Pacific herring (13). There are four genotypes related to geographic origin but increasingly more fish species are being infected (14). To date there is no commercial vaccine available. The G protein of VHSV, which is the only protein exposed at the surface, is the most immunogenic protein and experimental

DNA vaccines encoding VHSV- G have shown high levels of protection (15, 16). A recent innovation to further improve DNA vaccines is molecular adjuvants. In this strategy plasmid encoded signalling molecules are incorporated into a DNA vaccine-adjuvant construct to enhance anti-viral or inflammatory responses (17). Nevertheless, DNA vaccines face major hurdles to be authorized for marketing due to safety concerns regarding possible integration into an animal for food production, and inadvertent release into the environment of a plasmid coding antibiotic resistance (18). The latter may be solved by new methods of antibiotic free selection in *E.coli* (19). But alternative formats are being sought, particularly to deliver VHSV vaccines via mucosal routes. Promising results have been obtained with a PLGA encapsulated inactivated-VHSV vaccine via immersion and oral delivery to olive flounder (*Paralichthys olivaceus*) (20), which we discussed in Chapter 2 of this thesis.

A crucial step in novel vaccine development is to test indicators of protection. Here we test the capacity of our nanoparticle VHSV-G-frg16^{NP}, to induce specific antibodies against VHSV *in vivo* in a small scale trout infection model, as an initial step towards establishing a correlate of protective immunity (21). To our knowledge, this is the first report demonstrating viral proteins nanostructured as IBs can raise specific, functional, neutralizing antibodies in fish.

3. Materials and Methods

3.1 Fish

Rainbow trout (*Oncorhynchus mykiss*) juveniles purchased from a VHSV-free commercial fish supplier (Piszolla S.L., Cimballa Fish Farm, Zaragoza, Spain) were maintained in tanks at 14 ºC with a recirculating dechlorinated water system, light/dark regime of 12/12 h in the animal facility of the University Miguel Hernandez (UMH). Fish were fed daily a commercial diet of approximately 1% body weight and were acclimatized for 2 weeks prior to the experiment. Mean fish weight 2.2 ± 0.6 g and length 6.0 ± 0.6 cm.

3.2 Nanoparticles

The protein nanoparticle construct, VHSV-G-frg16^{NP}, contains the C-terminal half (amino acid residues 252–450) of the VHSV (07-71) G protein sequence (NCBI Genbank X59148) to the 3′-end, with the cysteine residues mutated to serine to assist expression in *E. coli*. The VHSV clone fragment 16 was originally described in (22). Figure 1 of that paper shows that the fragment encodes a major part of the high variability region of the G protein. Production as IBs, physical characterization and the innate anti-viral immune response to the nanoparticle *in vitro* is reported

in (8), which is chapter 1 of this thesis. A control nanoparticle, iRFP^{NP}, made of the immune-irrelevant near-infrared fluorescent protein (iRFP), iRFPHis cloned in pET22b (Genscript), was also transformed in *E.coli* BL21(DE3), and produced as IBs. The morphology of iRFP^{NP}, its emission spectrum and results of uptake studies in ZFL are shown in the supplementary material of this thesis, **Annex 1**.

3.3 Immunization and blood collection

Fish were anaesthetized in Tricaine (Sigma) 40 mg/L. Using insulin needles (BD microfine 0.3 mL, 30G), fish were i.p. injected with 50 µg of VHSV-G-frg16^{NP} or 50 µg of control nanoparticle iRFP^{NP} in a total volume of 30 µL phosphate buffered saline (PBS). A sham vaccinated group was injected with 30 µL PBS. Group/ tank distribution was as follows: Three tanks for infection: A) PBS n = 10 fish; B) iRFP^{NP} n = 10; C) VHSV-G-frg16^{NP} n = 12.Two other tanks D) VHSV-G-frg16^{NP} n = 8 (for blood sampling pre-infection), E) sentinel fish, n = 15 (5 fish for blood sampling pre-infection as untreated control sera, 10 fish as sentinels for survival without any treatment). Fish were then maintained for 30 days and were monitored for well-being and survival as an indicator of macro-toxicity of the nanoparticle. On day 30 post vaccination, fish from the 2 groups designated for blood sampling pre-infection were euthanized by Tricaine overdose (300mg/L) and blood was taken from the caudal vein with a 26G needle (BD Biosciences). Samples were stored at 4ºC to clot overnight, centrifuged at 3000 x *g* for 15 min at 4 ºC and serum was collected and stored at -80 ºC.

3.4 VHSV challenge

On day 31 post immunization fish in the 3 tanks designated for infection (PBS vaccinated, iRFP^{NP} vaccinated and VHSV-G-frg16^{NP} vaccinated) were i.p. injected with 30 µL of 3 x 10^7 median tissue culture infectious dose ($TCID_{50}$)/mL of VHSV-07.71 (23) propagated in EPC (fathead minnow *epithelium papulosum cyprini*) carp cell line, see (15) and references therein. Mortality was monitored until 23 days post infection (dpi), when remaining fish were euthanized and blood samples were taken as described above, to collect serum from infection survivors for enzyme linked immunosorbent assay (ELISA).

3.5 Confirmation of VHSV infection by viral titre in tissues of dead fish

Dead fish were stored at -20ºC. As soon as possible post-mortem (< 24 h) head kidney and spleen were dissected out and ground on ice in 50 µL culture medium (RPMI-1640 Dutch modified,

Gibco) + 2% fetal bovine serum (FBS) gamma irradiated (Cultek); 30 µL was used to obtain virus in supernatant for a $TCID_{50}$ titre assay. Tissue was dispersed in 500 µL RPMI + 2% FBS and passed through a 100 µm cell strainer (Falcon, Corning) then washed with 500 µL medium. Flow through was collected on ice and filtered (0.2 µm, Corning),and the supernatant was stored at -80 ºC. The same procedure was done with organ samples from survivor fish at day 22 post infection and, as a negative control, fish that had not been infected. To determine the viral titre, 100 µL of virus supernatant in quadruplet was used to infect confluent EPC cells in 1:10 serial dilutions from undiluted to 10^{-6}. Background control was EPC cells + 100 µL RPMI + 2% FBS. Cells were incubated at 14 ºC to allow the infection to develop. One week later, the supernatant was removed and 100 µL of a 0.05% crystal violet, 4% formaldehyde solution (Sigma) was added. Only live attached cells will stain (24). $TCID_{50}$ was calculated by the Spearman-Kärber method (25).

3.6 Inactivation and concentration of VHSV

To obtain UV-inactivated VHSV to coat ELISA plates, 40 mL of supernatant with VHSV titre 3.16 x 10^7 $TCID_{50}$/mL was exposed to UV-B at 1 J/cm2 using a Bio-Link Crosslinker BLX E312 (Vilber Lourmat, BLX-E312), as previously described (26) and stored at -80 ºC. Virus was concentrated by ultracentrifugation (Sorvall Discovery SE) using a Beckman 70Ti rotor at 35,000 rpm (125,000 x *g*) for 1 h at 4ºC. The pellet was suspended in 500 µL of Sigma water, semi-quantified by spectrophotometry at 280 nm (Nanodrop 1000) and stored at -80 ºC.

3.7 IgM antibody response (ELISAs)

To detect the presence of specific antibodies raised against VHSV in the trout sera, from immunized and control fish, both pre and post challenge, we performed indirect ELISAs. Pre-challenge samples were from VHSV-G-frg16^{NP} vaccinated and sentinel fish, and post-challenge samples were from survivors of VHSV-G-frg16^{NP} vaccinated and PBS vaccinated fish at the endpoint. iRFP^{NP} vaccinated fish were not included as there was only 1 survivor from that group. Briefly, Maxisorp 96 microwell plates (Nunc) were coated with inactivated VHSV at 0.5 µg/ well in 50 µL PBS overnight (O/N) at 4 ºC. All further steps were done at room temperature (RT) which was ~21 ºC. Washes were performed in triplicate with PBS + 0.05% (v/v) Tween 20 after all steps. Blocking was done with 3% skimmed milk in PBS for 2 h. Serum dilutions were prepared with PBS, 0.05% (v/v) Tween 20, 0.5% BSA. Serum dilutions were added in duplicate at 100 µL/ well and incubated for 2 h followed by washes in triplicate. Monoclonal primary antibody anti-trout IgM was produced and isolated in house from the mouse hybridoma clone 1.14 (27). The optimized working concentration was 100 µL/ well at 1:2000 diluted in wash

buffer. The secondary antibody was HRP conjugated goat anti-mouse IgG (Sigma A4416) 100 µL/ well at 1:2000 diluted in wash buffer. Detection was via 3,3'5,5' tetramethylbenzine (TMB) substrate reagent set (BD Biosciences), 100 µL/ well, incubating in the dark for 30 min. The reaction was quenched with 100 µL of 2M H_2SO_4. Absorbance was measured at 450 nm on a spectrophotometer (Victor 3, PerkinElmer). The specific antibody titre was defined as the inverse of the greatest dilution which still gave a positive result.

3.8 VHSV neutralization assay

To determine the neutralizing capacity of the sera in fish vaccinated with VHSV-G-frg16^{NP} prior to challenge vis-a-vis non-vaccinated, a neutralization assay was performed based on (15). Briefly, fish serum was complement-inactivated at 45ºC for 30 min. Serum dilutions were incubated for 3 h at 14ºC with virus at multiplicity of infection (MOI) 3 x 10^{-2} in a 1:10 volume ratio, diluted serum:virus (30 µL serum dilution, 300 µL virus). Then 1% volume of healthy trout serum was added as a source of complement and the mixture was incubated for a further 30 min. The mixture (100 µL/ well in triplicate) was used to infect confluent EPC cells in 96 well plates. Non-serum controls were the same virus preparation mixed, incubated and used to infect EPCs as above with 1) a strongly neutralizing monoclonal antibody (MAb) 3FIA2 against the VHSV glycoprotein (28), but without addition of complement; 2) PBS and 1% volume of healthy trout serum as complement source; and 3) the virus alone. Infected cells were incubated for 2 h at 14ºC, washed with PBS, and then incubation continued for 24 h in fresh culture medium (RPMI + 2% FBS). Cells were fixed with 4% formaldehyde (Sigma) and ice cold methanol, followed by an immunostaining focus assay adapted from (29) using primary antibody MAb 2C9 (30) and secondary antibody GAM-FITC (Sigma 4600042). Viral infection foci were detected on an IN Cell Analyzer 6000 imaging system (GE Healthcare Life Sciences). Images were processed with Fiji open source image processing package (31).

3.9 Statistics

Graphs and analyses were performed with Prism 6.01 software (GraphPad). Data are shown as mean ± standard error of the mean (SEM) and $p < 0.05$ was considered statistically significant.

3.10 Ethics statement

In vivo and *in vitro* experiments with trout and live VHSV were performed at the "Instituto de Biología Molecular y Celular" (IBMC), and the animal facility of Universidad Miguel

Hernández de Elche under the supervision of Dr Maria del Mar Ortega-Villaizan. All methods were performed in accordance with the Spanish and European regulations (RD53/2013 and EU Directive 2010/63/EU) for the protection of animals used for research experimentation and other scientific purposes.

4. Results

4.1 Production of specific anti-VHSV IgM

Trout vaccinated with VHSV-G-frg16^{NP} clearly induced a specific antibody response by 30 days post i.p. immunization, with a pre-challenge titre of 240 (**Figure 2A**). Moreover, 23 days post VHSV challenge, a substantial specific antibody expansion was observed with antibodies still detectable at 8 times this dilution (titre 1920). The specific antibodies detected in VHSV-G-frg16^{NP} vaccinated fish significantly surpassed antibodies raised due to the VHSV infection alone in the sham (PBS) vaccinated group. This is demonstrated by a statistical analysis of results for the 1:30 dilution in **Figure 2B**. In addition, vaccinated fish had consistently high levels of antibody, whereas the antibody response to infection was more variable in the PBS vaccinated group, see error bars in both **Figure 2A and B**.

4.2 Macro-toxicity, Infection and Survival

During the 30-day period post immunization with VHSV-G-frg16^{NP} there was 1 death on day 25 post administration from a total of 20 fish vaccinated, while all others vaccinated showed no signs of malaise. The 10 fish vaccinated with iRFP^{NP} also remained healthy in appearance and behaviour. This is in line with our other studies using these and other IBs in zebrafish, in which no detrimental effects have been observed (6, 8). We therefore consider this death was incidental and the nanoparticles are safe in terms of macro-toxicity. Post-infection, the presence of virus was confirmed in fish which died at the peak of infection, between 5 - 9 dpi. They had enlarged spleens and we detected a viral titre of $TCID_{50}$ /ml = 1.5×10^5 for sham vaccinated fish and 5.6×10^3 for vaccinated fish. Neither negative controls, fish which died later (the first death in iRFP^{NP} vaccinated fish was at 18 dpi), nor survivors at 22 dpi showed presence of virus, presumably since at this point the infection was not detectable by this method, had cleared, or had not developed in some of the survivors. As only 1 fish that was VHSV-G-frg16^{NP} vaccinated died at the infection peak, the data is very limited, but the difference in titres is consistent with vaccinated fish having less viral load.

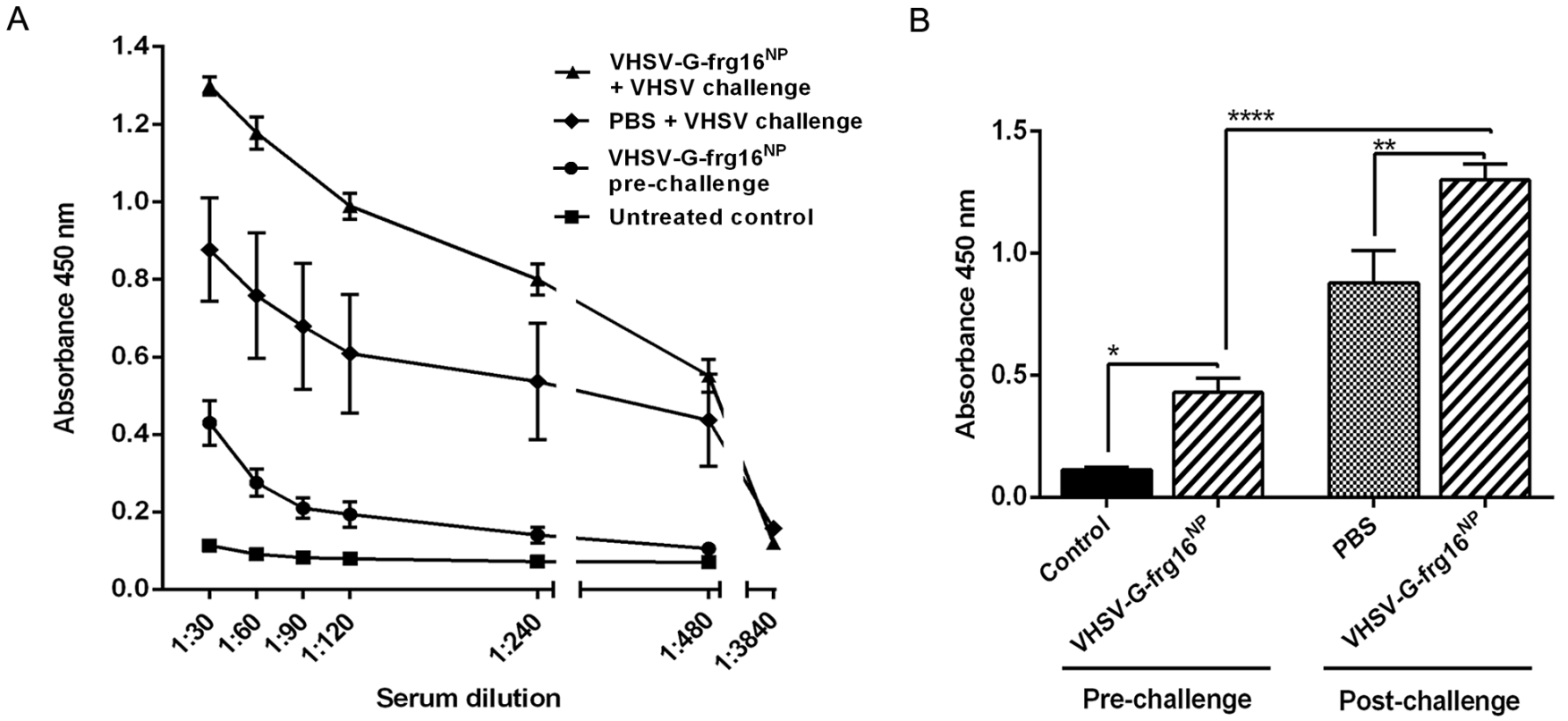


FIGURE 2: Presence of specific anti-VHSV IgM in sera of rainbow trout juveniles immunized with protein nanoparticle VHSV-G-frg16^{NP}.

A. ELISA titration curves using inactivated VHSV as antigen. Trout were vaccinated i.p. with 50 µg VHSV-G-frg16^{NP} or 30 µL PBS & left for 30 days until VHSV challenge. Sera for ELISA collected from: untreated control (■); VHSV-G-frg16^{NP} vaccinated, pre-challenge (●); PBS vaccinated & challenged with VHSV, infection survivors at 23 dpi (♦); VHSV-G-frg16^{NP} vaccinated & challenged with VHSV, infection survivors at 23 dpi (▲). Absorbance readings were measured at 450 nm. Error bars indicate standard error of the mean (SEM), n=4. dpi = days post infection.

B. Comparison of specific anti-VHSV IgM in sera at 1:30 dilution using data from Fig 2A. Error bars indicate standard error of the mean (SEM), n = 4. Statistical analysis with a one-way ANOVA & Tukey's multiple comparisons test. Significance levels *p < 0.05; **p < 0.01; ****p < 0.0001.

The survival results, **Figure 3**, were also limited by a low number of deaths in the PBS vaccinated group, however 90% of the control nanoparticle group died. The VHSV titre we injected i.p. was based on published results (32), (33), adjusted according to our fish size and our aim to seek cumulative mortality, but virulence was less than expected in the sham vaccinated group. An infection of greater virulence would be required to discriminate better between vaccinated and unvaccinated fish in terms of survival (21). Unfortunately, two thirds of the tank water was lost through leakage from the PBS sham vaccinated tank during the night of 8 dpi, and water had to be replaced twice before repair. Thus, horizontal transfer in this control group was minimized at a critical point, which may explain why less deaths occurred. Nevertheless, the relative percentage survival (RPS) measured by [1 - (% cumulative mortality of experimental group / % cumulative mortality of control group)] x 100, was consistent with vaccinated fish having an advantage: RPS of VHSV-G-frg16^{NP} vaccinated fish was 55% with respect to PBS sham vaccination and 90% with respect to the control nanoparticle group.

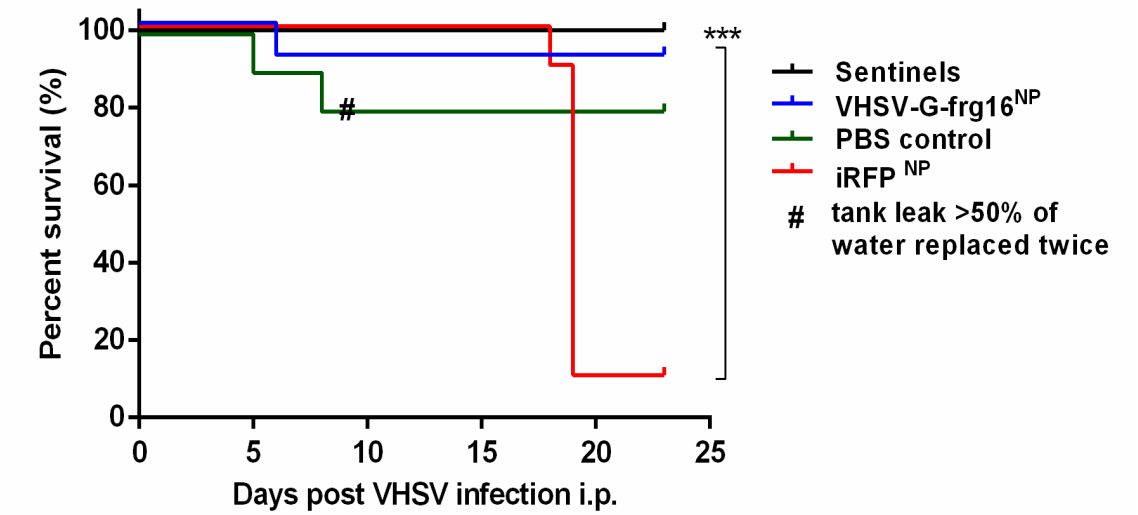


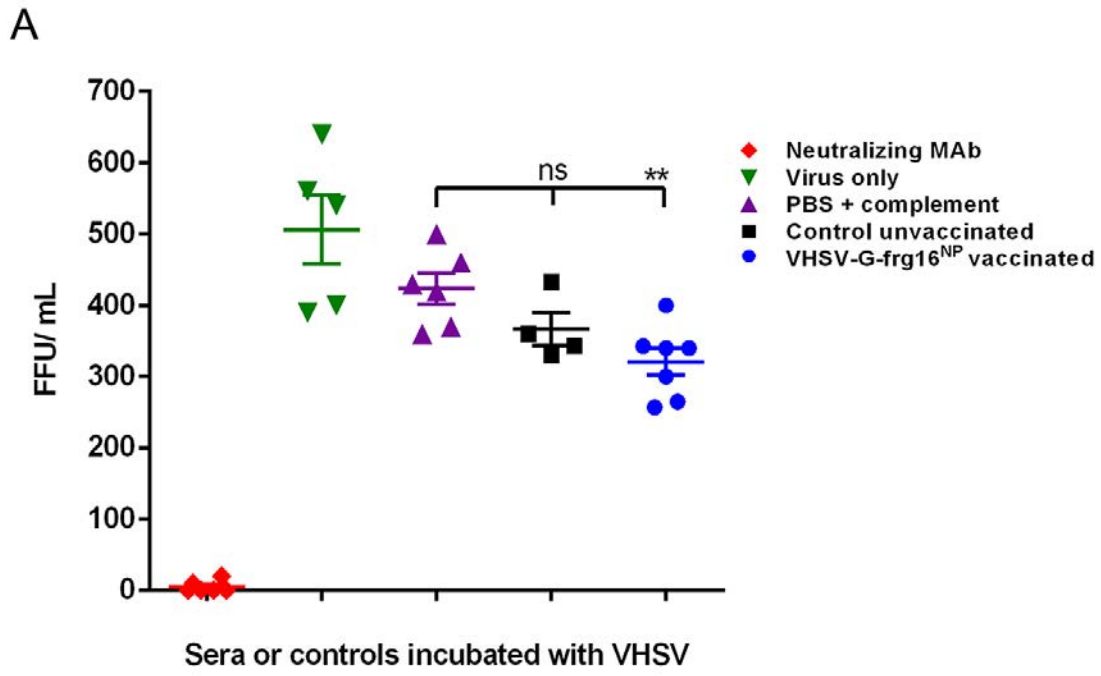
FIGURE 3: Survival curve of trout immunized with VHSV-G-frg16^{NP}, challenged with VHSV.

Fingerling trout, 30 days after i.p. injection with: 50 µg VHSV-G-frg16^{NP} (n = 12) (**blue**); 50 µg iRFP^{NP} control (n = 10) (**red**); or with PBS (n = 10) (**green**); were challenged with i.p. injection of 30 µl of 3 x 10^7 $TCID_{50}$/ml viral haemorrhagic septicaemia virus (VHSV). Significant differences were analysed using the Log-rank test ***, $p < 0.001$. Sentinels were untreated, uninfected controls (**black**).

4.3 Neutralizing assays

To see if antibodies raised by VHSV-G-frg16^{NP} immunization could inhibit VHSV activity, a neutralization assay was performed. Only the sera of vaccinated fish pre-challenge and untreated controls were used. In this way, neutralizing antibodies, if present, could be directly linked to vaccination. **Figure 4A** shows the viral foci formed when diluted sera (1:25) were incubated with VHSV. Compared to the control (virus incubated with PBS before adding complement), the sera of VHSV-G-frg16^{NP} vaccinated fish was able to decrease virus infection of the cells significantly,

while sera from unvaccinated control fish did not significantly decrease viral infection. There was however some overlap between groups, showing a range of neutralizing capacity among individual fish sera and their antibodies. **Figure 4B** provides representative examples of the VHSV focus forming units found in treatments with PBS control and in sera from VHSV-G-frg16^{NP} vaccinated fish. In addition to greater numbers of foci, larger, expanding foci were found in the control groups.



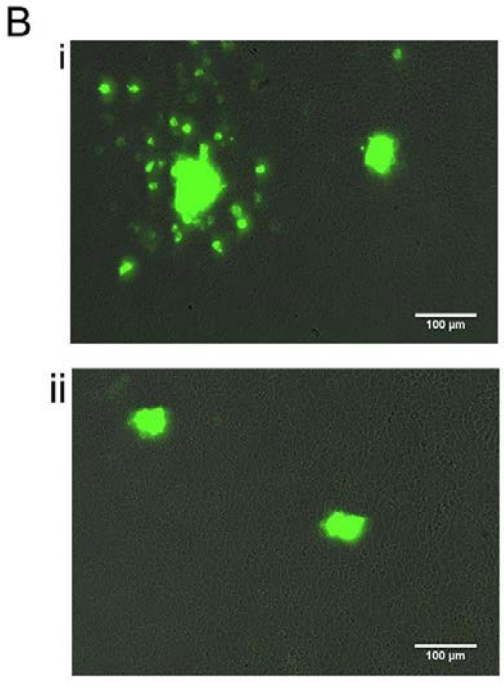


FIGURE 4: Neutralizing capacity of sera from fingerling trout 30 days post immunization with protein nanoparticle VHSV-G-frg16^{NP}.

A. Neutralization assay. Viral focus forming units (FFU) were detected using an immunostaining focus assay. Graph shows FFU/ mL using virus mixed with sera diluted at 1:25 from: VHSV-G-frg16^{NP} vaccinated (●); & unvaccinated control fish (■); compared to virus mixed with PBS and complement (▲), in parallel. Error bars indicate standard error of the mean (SEM), minimum n = 4 fish. Horizontal bar = mean. Statistical analysis: one-way ANOVA with Dunnett's multiple comparisons test against the PBS + complement control group. Significance level **p < 0.01, ns = not significant. Technical experimental controls were virus mixed with: 1:50 dilution of neutralizing MAb (♦); & virus alone incubated without complement (▼), used to infect EPCs.

B. Representative images of viral FFU in treatment groups described in Fig 4A. Images are brightfield & FITC merged i) PBS + complement control ii) VHSV-G-frg16^{NP} vaccinated.

5. Discussion

We have demonstrated here that the protein nanoparticle VHSV-G-frg16^{NP} induces specific antibodies against VHSV in fingerling trout, without the use of any adjuvant. The ELISA results show a clear induction of specific antibodies 30 days post immunization, with a large, consistent expansion post infection. Moreover, the neutralization assay indicates the presence of neutralizing antibodies among those raised by vaccination. We have achieved these results by injecting the immunogenic protein in the form of *insoluble* IBs. VHSV-G-frg16^{NP} recombinant protein is cheap, robust and simple to produce for safe use in fish (8). As a *soluble* protein, the immunogenicity of VHSV protein G frg16 has been demonstrated by pepscan mapping (34) and by antibody binding studies using sera from VHSV infection survivors (35). But our new configuration overcomes the difficulties faced in making labile soluble proteins into viable vaccine formats, without any need for encapsulation. Here, VHSV-G-frg16^{NP} released enough bioactive native-like protein from within the network of IB amyloid aggregate, to induce *in vivo* specific antibody production. This mechanism of slow release of functional protein by IBs is currently being explored in applications for human medicine (36). Here we show the use of IBs to raise virus-specific antibodies *in vivo* as an antiviral vaccine strategy for fish.

Concerning immunogenicity, it is of interest for design that VHSV-G-frg16^{NP} induced antibodies which showed virus neutralizing capacity, despite having cysteines mutated to serines in order to facilitate production in *E.coli*. Note that cysteines on the protein surface are one of the three signature amino acids for antigenic determinants (37) and are strongly implicated in tertiary structure. Results from the neutralization assay showed that in the mixed population of antibodies raised, there was significant decrease in VHSV infectivity in the sera of vaccinated fish. Inhibition of VHSV infectivity by a similar assay has been reported using fish sera vaccinated with a DNA vaccine encoding the G protein with 2-4 added CpG motifs (38). We found not only were there less focus forming units when the virus was incubated with sera from VHSV-G-frg16^{NP} vaccinated fish, but also very large foci were primarily found in the PBS control and the unvaccinated control groups, as well as in the virus only technical control (**Figure 4B**). This indicates more virus propagation in the control groups. There was some overlap in the neutralizing activity of certain serum samples of unvaccinated and vaccinated fish (**Figure 4A**). Interestingly fish, as other vertebrates, have natural antibodies that are present without apparent antigenic stimulation and in trout they can partially neutralize VHSV (39). However, the number of focus forming units per mL for virus mixed with the sera of unvaccinated fish was not significantly different from that of virus mixed with the PBS control.

As mortality in the viral challenge did not sufficiently discriminate between vaccinated and unvaccinated fish we are unable to correlate virus-specific antibody production with protection

(21). Optimization of dose, immunization regime, the challenge model and exploration of alternative delivery routes is work for the future. This is a small scale, but crucial, proof of concept study. We show in trout fingerlings, VHSV-G-frg16^{NP} induces the production of specific anti-VHSV antibodies which are functional against VHSV, as a surrogate of protection. The work here demonstrates *in vivo*, the potential of IBs made of antigenic viral proteins as a viable, practical bio-material for the development of novel vaccines in aquaculture.

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GENERAL DISCUSSION

1. Inclusion body production and proof of concept tests

1.1 Nanoparticle production

As a new fish vaccine strategy, our aim was to produce and test viral antigen recombinant protein nanoparticles structured as IBs. The IB format was chosen due to its stability, safety and bioactivity as a slow release carrier and immunostimulant; as well as because of the simple production procedure involved. In Chapter 1 we demonstrated the successful production of IBs in *E. coli* BL21(DE3) of the 3 target antigenic proteins: IPNV viral protein 2, VNNV coat protein and VHSV-G (from amino acids 252-450), called VHSV-G-frg16. The first two proteins are viral capsid proteins, whereas VHSV-G-frg16 is the C-terminal half of a viral glycoprotein. Viral capsid proteins are known to self-assemble into highly ordered particles, which is indeed the basis for producing viral-like particles (VLPs) (1). We were able to produce the capsid protein IBs IPNV-VP2^{NP} and VNNV-C^{NP} as full-length constructs. This approach aimed to increase the probability of maintaining native epitopes. However, the VHSV-G protein proved to be toxic for *E. coli*. To solve this, rather than opting to change the strain (2), for example to BL21(DE3)pLysS, we tried a more fundamental change, testing several modified fragment constructs of the G protein (3). The nanoparticle we successfully produced in high yield, VHSV-G-frg16^{NP}, was half the full-length sequence, with removal of the hydrophobic transmembrane region and with cysteines reduced to serines to facilitate expression in *E. coli*.

To generalize these findings for future work in terms of design, the choice of the immunogenic protein will influence whether IBs can be readily obtained. If the protein is not aggregation prone various strategies can be employed. The production temperature can be increased to 40-42 ºC (4), though the effect of this on functionality for our purposes is not known. Alternatively, a short sequence may be fused to a polypeptide to promote IB formation. Examples of these so called "pull-down peptides" such as the VP1 protein from foot-and-mouth disease, can be found in Table 2 of (4). A VP1-GFP construct produced as an IB retained activity as a fluorophore, indicating the polyprotein was functional (5). From our experience, the two fish capsid proteins produced in this study could be candidates for use in this manner and may be of interest in a multivalent IB finfish vaccine construct as an aggregating sequence as well as antigen. Concerning the VHSV-G protein, if other rhabdoviruses were to be targeted for vaccine development by our strategy, then a comparable construct to VHSV-G-frg16^{NP} could be considered. All rhabdoviruses have an external G protein (6) and sequences of teleost G proteins for IHNV and SVCV are publicly available in uniport.org. As discussed in Chapter 3, it was of great interest to us that VHSV-G-frg16^{NP} retained sufficiently immunogenic epitopes to raise specific, neutralizing antibodies against VHSV in trout, despite the modifications to the protein sequence, the lack of glycosylation

in prokaryote production and the format as an inclusion body. These results validated the approach.

In terms of scalability, there is a focus area in heterologous protein production via microbial cell factories very relevant to us: Designing methods to recover large quantities of functional *soluble protein* from IBs by mild extraction protocols (7, 8). That is to say, scalable production and isolation of IBs is already developed as the first step in this process. Compared to methods to obtain and purify soluble protein our production and isolation protocol is very simple and practical. Moreover, the IB product is stable and can be lyophilized, retaining functionality (9). The advantages in terms of production, storage and transport are evident.

1.2 Safety

As regards safety, the use of recombinant proteins avoids the critical issues related to DNA vaccines such as potential integration into the host genome, which, however unlikely, is still unclear (10). There is also concern about environmental release during the vaccination process, particularly considering antibiotic resistance genes may be used for selection (11). Even though the DNA vaccine CLYNAV has been approved, the EU Commission guidelines state that future DNA vaccine applications will be considered on a case by case basis. Each different plasmid backbone and the transgene are a potential risk (10). Another issue is whether the vaccinated fish should be classified as a genetically modified organism (GMO), which is linked to whether integration could occur (10, 12). IBs do not contain intact, functional genetic material, but rather some bacterial nuclei acid debris as remnants from the simple purification process. This would be rapidly degraded *in vivo* and the antigen, being just protein, does not imply any risks in this sense. This simplifies greatly safety issues for vaccine development.

Concerning toxicity, the tests we have performed so far with our nanoparticles (or "nanopellets", NPs) are preliminary. We did an MTT assay for cytotoxic and cytostatic effects *in vitro* (Chapter 1) and we monitored survival and well-being in 60 vaccinated Senegalese sole juveniles after both injection and intubation of NPs for up to 45 days (Chapters 2) and 20 vaccinated trout juveniles for 30 days (Chapter 3). To date, we have seen no signs of toxic effects, but obviously we need to keep in mind Directive 2001/82/EC, which provides guidelines in the section "Requirements of Immunological Veterinary Medicinal Products" to ensure our approach is viable. For example, to fulfil requirements, more information will be needed about the nanoparticles' biodistribution and persistence (11). We do not know yet how long the inclusion bodies persist in the fish. The indications we have are that in ZFL, the NPs taken up by cells start to be metabolized between 24 and 48 h, as the mean fluorescence intensity/cell began to drop during this time (Chapter 1). In the *in vivo* oral uptake studies in zebrafish (Chapter 1) we found maximum intestinal uptake was

achieved by 6 h for all 3 NP constructs and by 48 h it was negligible for VNNV-C^{NP} and VHSV-G-fr16^{NP} and minimal for IPNV^{NP}. Other information comes from previous histology studies on IB uptake in zebrafish gut tissue and in rainbow trout. In zebrafish, a stomachless fish, post oral gavage of TNFα IBs, nanoparticles were already being taken up in epithelial cells and seen in the lamina propria 1 h after oral intubation, while by 24 h they were no longer observed in intestinal tissue (13). In trout, 24 h after oral gavage of TNFα IBs to the stomach, nanoparticles were found in the pyloric caeca, mainly at the villi base and submucosa, as well as in the midgut, where they were seen in the villi apex and lamina propria (9).

However, we do not know how long the IB persists *in vivo,* nor how long the slow release of protein occurs, nor whether there is any temporary sequestration in the animal or undesirable effects on the immune system. It is important to note our NPs are free from viable *E. coli*, which implies no accumulation of bacteria in the fish, nor release to the environment. This could be an issue with a recently reported promising strategy for a RGNNV vaccine that uses whole live *E. coli* transformed with a plasmid containing the antigen gene and ampicillin resistance. The bacteria are incorporated into fish food pellets as an adjuvant and vaccine carrier (14).

1.3 Immunostimulant properties

The immunostimulant, or adjuvant properties of our NPs were demonstrated *in vitro* in both ZFL and RT-HKM primary cells cultures as they triggered the innate anti-viral immune response (Chapter 1). We consider this is due to the viral protein carried, but also the repetitive aggregation of the IB structure may play a role. Note the repetitive, particulate structure of VLPs can stimulate the innate adaptive response and promote uptake by antigen presenting cells (15). Indeed, VLPs have been described as "self-adjuvating" (16). We do not know how far this could apply to viral proteins nanostructured as IBs. But we obtained a particularly high anti-viral innate immune response in ZFL stimulated by the capsid protein VNNV-C^{NP} (discussed in Chapter 1). An innate inflammatory response is also expected from the IBs produced by our method due to remaining bacterial impurities. For instance LPS has been quantified for IB constructs in various bacterial strains and differs accordingly (5). It may also vary between batches of the same construct and strain. In addition the *in vitro* stimulus of various pro-inflammatory related genes such as *TNFα*, *il1-β* and *cox-2* was demonstrated for our control nanoparticle, iRFP^{NP} in the same paper (5). We would expect a comparable, non-protein specific upregulation of pro-inflammatory genes induced by other IBs produced by our method. Importantly, in this current work, iRFP^{NP} did not stimulate an anti-viral innate immune response, showing the importance of the viral antigen to achieve this (Chapter 1). The crucial result is that our viral antigen NPs have immunostimulant properties. This opens up the possibility of avoiding adjuvant use which would save production costs and

possible adverse effects (17), but may be necessary for a stronger response, especially via the oral route (18). Protein subunit vaccines typically have two hurdles: instability and degradation *in vivo* and the necessity for adjuvant to induce a sufficient response (19). The first has clearly been overcome by our approach and for the second, our results are encouraging, though by no means definitive.

Once we had produced the nanoparticles and demonstrated uptake and general safety *in vitro* and *in vivo* in zebrafish, as well as immunogenicity *in vitro*, the proof of concept phase was completed. Without further studies on detailed characterization of the particles or mechanisms of action, we moved directly to farmed-fish models to test indicators of protection. This was a deliberate strategy to speed up translation of research to industry, in line with the concept of goal oriented, rational use of resources.

2. Tests in farmed fish species

2.1 Nanoparticles used and relevance

Undoubtedly the most exciting part of this work has been testing the nanostructured proteins in target farmed species. We were able to successfully carry out studies with two of the NPs, VNNV-C^{NP} in Senegalese sole and VHSV-G-frg16^{NP} in rainbow trout. It is unfortunate the testing of IPNV^{NP} was not logistically feasible at this stage. We had included IPNV as a target virus because of its relevance to the aquaculture industry and, in contrast to the other viruses we chose, there are a number of vaccines in the market. This includes oral and immersion vaccines, though these are only marketed in Chile. In Europe only injectable vaccines are commercially available (see General Introduction Table 4). Extensive information about IPNV is published (20) and the virus is known to infect through the intestine (21) as well as the gills and skin. We considered it a good virus to target for an oral vaccine and for which we would have comparative information. Nevertheless, the other 2 viruses we targeted are more relevant in the sense that the need for vaccines is far more pressing. For VHSV there are no commercially available vaccines and for VNNV only one injectable vaccine. Moreover, this vaccine, ALPHA JECT micro 1 Noda, is not indicated for the emergent strain RGNNV/SJNNV we have focused on.

The most significant contribution of this thesis was to demonstrate that our NPs could raise specific antibodies against the virus *in vivo* in relevant farmed fish species. Specific antibodies are considered to be very reliable correlates of protective immunity for fish vaccines (22). Here we have measured them as a surrogate of protection (23), and they are a key indicator of the potential of this vaccine approach. Suffice to say, we would not proceed with future challenge models without detecting specific anti-viral antibodies at this stage.

2.2 Vaccinating Senegalese Sole with VNNV-C^{NP}

In *S. senegalensis* we saw that juvenile fish injected with VNNV-C^{NP} without adjuvant, raised specific antibodies against the viral coat protein (anti-VNNV-C IgM) by 17 days post vaccination (dpv), similar for both doses of nanoparticle (50 and 500 µg/ fish). The specific antibody titre expanded impressively with booster, reaching higher levels with the higher dose (Chapter 2, Figures 6 and 7). An expansion of this nature was very recently reported by Gonzalez-Silvera, 2019, using lysed bacteria transformed with a plasmid encoding the RGNNV capsid protein as an i.p. injected vaccine in juvenile sea bass. No booster was given, the expansion was seen 30 dpv, 2 days post challenge, achieving a high RPS (14). In our scenario, young sole injected with VNNV-C^{NP} were very capable of raising antibodies against the NNV capsid protein and strong immune memory was evident with the booster. The next step would be to perform a challenge both with and without booster and compare survival, as considering the results obtained in sea bass, a booster may not be needed. However, as discussed in Chapter 2, heterologous boosting is more likely to induce both humoral and cell-mediated immune responses (18, 24). Moreover, as we now know VNNV-C^{NP} is immunogenic and can raise viral capsid specific antibodies when injected, it would be of interest to test a combination of delivery routes as a more practical strategy for the farmer. For example, injection of VNNV-C^{NP} plus oral booster followed by challenge, or immersion and then oral booster. The immersion route should be investigated, as the highest mortality from VER occurs in larvae and juveniles. Immersion is a practical method provided the fish are small enough and the vaccine is not expensive, as quite large amounts are required.

Ideally, oral delivery in the feed, would be the low cost, low stress, low tech method of choice for the farmer. We explored this by administering VNNV-C^{NP} at both low and high dose in parallel by i.p. injection and oral intubation (Chapter 2). We used oral gavage for these first trials as there will always be differences in the amount each individual eats and in this way, we could uniformly deliver precise quantities to the intestine. We found a low level of specific antibodies raised after oral delivery compared to injection and a small increase in antibodies after oral booster with the low dose and what we presume is tolerance with the high dose. Two things are clear. First, for the oral vaccination route to work, optimization of the dose and administration regime is critical to avoid tolerance or anergy. There is a lot of variation in oral administration regime strategies in the literature. Essential ground work needs to be done with a series of doses and timing of feed regimes for each vaccine formulation and context. Work of this nature has been reported by Chien 2018, administering VLPs formed by orange spotted grouper NNV to grouper larvae (25), discussed in Chapter 2. Second, the immune response via the oral route may be suboptimal compared to injection. However, the enormous practical and cost-saving advantages of an orally delivered vaccine can outweigh this drawback. It is a matter of optimizing format, dose regime and adjuvant use. Several authors who compared injection and oral delivery in parallel have

emphasized the significance of getting an antibody response and protection via the oral route, though the immune response was less potent than via injection (25, 26). We definitely need to explore this further and incorporate challenges to determine the degree of protection achieved.

The gene expression results performed in *S. senegalensis* on fish injected with VNNV-C^{NP} showed upregulation of *cd4* and *IgM* in the head kidney (Figure 7, chapter 2). According to Munang'andu and Evensen 2015, this is a typical pattern for non-replicative vaccines such as inactivated whole virus (IWV) and subunit vaccines. It indicates a systemic humoral immune response in which the effector mechanism against the pathogen is antibody neutralization of virus (27). Replicative vaccines such as live attenuated and DNA vaccines additionally induce cellular immune responses, which can be inferred by upregulation of *cd8 (27)*. We did not detect upregulation of *cd8* in any of the tissues tested, which is consistent with this interpretation. It should be highlighted that most commercial vaccines in aquaculture are non-replicative because of their safety compared to DNA and live vaccines (18). As regards oral delivery of VNNV-C^{NP}, gene expression results showed, above all, an upregulation of *IgT* in the intestine, indicative of a mucosal immune response, but also some *IgM*, noting that IgM+ B cells have been reported in the teleost gut (28).

2.3 Vaccinating rainbow trout with VHSV-G-frg16^{NP}

In the experiments with *O. mykiss*, we were able to work with viral infection models, which added a further dimension to the results we could obtain. However, we only had permission to inject the fish, so work with oral gavage was not performed. In trout we saw that fingerling fish injected with VHSV-G-frg16^{NP}, without adjuvant, raised antibodies against the virus. Upon infection with VHSV, the anti-VHSV IgM titre expanded impressively and was significantly higher than in unvaccinated infection survivors (Chapter 3 Figure 2, A and B). We therefore demonstrated clearly the potential of the nanoparticle to stimulate an adaptive response in trout.

The results concerning infection survival and viral load were consistent with the NP providing protection. However, an infection with greater virulence is needed to distinguish better between vaccinated and unvaccinated fish in terms of survival (22). Importantly, in trout we were able to demonstrate that the specific anti-VHSV IgMs raised after injection of our nanoparticle could reduce VHSV infectivity. Therefore, VHSV-G-frg16^{NP} elicited the production of functional, neutralizing antibodies. The neutralization was moderate, but significantly greater than controls. Concerning this result, we should consider the following: Delivery was a single injection (50 µg/fish) with no adjuvant. The nanoparticle is a modified version of half of the VHSV-G protein. Antibodies raised will be a mixed population. And, as a non-replicating vaccine, exposure of antigen to APCs and their subsequent activation is limited to the dose administered (27). We were

therefore very encouraged that neutralizing antibodies could be detected without booster. We think that the slow release of antigenic protein provided by the IB format could be prolonging exposure time of APCs to the antigen and, also, the immunostimulant properties of the bacterial impurities carried can help APC activation. This result further validated our design approach.

Table 1: **Comparison of basic characteristics of our strategy with DNA and IWV vaccines**

Vaccine type	Viral antigen inclusion bodies (our NPs)	DNA	Inactivated whole virus (IWV)
Cost	Cheap, recombinant protein production	Cheap, plasmid design & production	Expensive, culturing of virus, use of adjuvant
Delivery method	Injection i.p., oral (needs more testing), immersion?	Injection i.m. OR oral route, but requires encapsulation	Injection i.p. OR oral route, but requires encapsulation
Type & uptake	Non-replicative, endocytosis, phagocytosis of antigen by APCs	Replicative, penetration of host cell membrane, cytoplasmic replication	Non-replicative, endocytosis, phagocytosis of antigen by APCs
Response	Humoral, antibodies	Humoral and cellular, antibodies & cytotoxic lymphocytes	Humoral, antibodies
Safety / Practicality	Safe, easy to produce, could be mass delivered in feed or injected in PBS	Safety unclear, difficult to license; i.m. injection route most studied	Safe, but injection of individuals is labour intensive or requires sophisticated, expensive machinery. Oil adjuvants improved but not ideal
Main drawbacks	So far, oral route shows weak immunogenicity. Optimization needed. Adjuvant required? Booster likely required	Safety analysis & licensing is a case by case basis. Consumers' may have negative attitude to a perceived GMO.	Expensive to individually vaccinate fish. Only feasible for high market value fish
Main advantages	Cheap, practical, safe for the fish and environment.	Good protection achieved by i.m. delivery against some viruses	Good protection by i.p. delivery for several viruses. Multi-valent vaccines against virus & bacteria in the market
Comments	Good levels of specific, functional antibodies raised by i.p. delivery. Further oral route studies, heterologous boosting & challenges needed. Immersion should be tried for very small fish & larvae.	Only two DNA vaccines have been licensed for aquaculture. Safety and ethics are barriers for approval of further vaccines	This approach continues to be the main commercial method. But cheaper more practical vaccines are needed. Almost all vaccines are just for salmon, or salmon & trout.

Table 1 provides a brief summary, comparing the main aspects of our approach with DNA vaccines and IWV, the most common commercial vaccine type currently available. Table 1 draws on information from (10, 27, 29) and our results.

3. Methodological contributions of this thesis

This project has pioneered various methods which should be highlighted. First, we have performed a comprehensive study about using bacterial inclusion bodies as transporters and slow releasers of functional fish viral protein antigens to elicit innate and adaptive immune responses *in vitro* and *in vivo*. Next, we have developed oral gavage techniques as a means of introducing precise amounts of any compound of interest in suspension or solution to the intestine of small fish, without injuring the animals. In zebrafish, this work was initiated in our lab by Dr Jie Ji and further used here, resulting in a publication in the Journal of Visualized Experimentation, 2018 (30). In Senegalese sole, the oral intubation method is new, and our results demonstrate that it can be successfully applied in juvenile flatfish. We also contribute further tools for *S. senegalensis*, a less studied species than trout. We provide a set of PCR primers for adaptive immune system marker genes with qPCR efficiency studies. We also have developed a specific ELISA for detection of anti-NNV antibodies in Senegalese sole, based on the clone we designed for the capsid protein of the emerging strain of betanodavirus RGNNV/SJNNV. From the same clone we were able to produce soluble purified protein to coat ELISA plates and the insoluble protein as inclusion bodies for vaccination, by modifying the culturing conditions.

We hope others may take advantage of all these tools.

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CONCLUSIONS

The conclusions of this thesis are summarized below:

1. Antigenic proteins from three viruses significantly affecting European farmed finfish were successfully produced as bacterial inclusion bodies in *E. coli* BL21(DE3). They were semi-purified to maintain functionality and immunostimulant properties and physically characterized by FESEM. These stable, bioactive protein nanoparticles or "nanopellets" (NPs) were: VNNV-C^{NP}, IPNV-$VP2^{NP}$ and VHSV-G-$frg16^{NP}$; corresponding to the coat protein of the emergent strain of the viral nervous necrosis virus (RGNNV/SJNNV), viral capsid protein 2 of the infectious pancreatic necrosis virus (IPNV), and a C-terminal fragment of the G glycoprotein of the viral haemorrhagic septicaemia virus (VHSV amino acids 252-450). The first virus affects marine fish such as sole, turbot and sea bream; and the latter two affect salmonids.

2. All three nanoparticles were taken up *in vitro* by zebrafish liver (ZFL) cell line, using fluorescently labelled NPs. Flow cytometry showed uptake at 6 h post addition of NPs to the culture medium, the earliest time tested. This continued over the first 24 h. By 48 h some metabolism of the NPs was apparent. Confocal microscopy confirmed that the NPs were inside the ZFL cells. No toxicity was observed in an MTT test with ZFL.

3. The NPs upregulated gene markers of the anti-viral innate immune response *in vitro* (interferon stimulated genes) in ZFL, and in primary trout macrophages, as demonstrated by qPCR. They therefore have immunostimulant properties.

4. All three NPs were taken up *in vivo* by zebrafish through the intestine when administered by oral gavage. Maximum uptake had already occurred by 5 h post intubation as determined by flow cytometry of intestinal cells. No toxicity was observed up to 48 h post intubation.

5. In farmed fish models using Senegalese sole (*S. senegalensis*) juveniles, VNNV-C^{NP} administered in low and high doses (50 and 500 µg) by i.p. injection raised specific antibodies against the RGNNV/SJNNV viral coat protein, determined by ELISA. These antibodies expanded considerably post booster. Gene expression results indicated a systemic humoral response typical of non-replicative vaccines. This nanoparticle is therefore immunogenic *in vivo* when injected without adjuvant to a farmed species affected by the virus.

6. When VNNV-C^{NP} was administered by a novel oral gavage method to *S. senegalensis* only low levels of antibodies were raised. These expanded weakly post booster with the low dose and dropped with the high dose indicating a tolerance response for the high dose. Gene expression showed *IgT* was upregulated in the intestine as a mucosal immune response. More work is needed to optimize the response via the oral route and adjuvants may be required. The immersion route should be tested as well as heterologous boosting. Challenge experiments are needed to determine the protection conferred via all routes. Encouragingly, no toxic effects were observed over a period of 45 days neither by i.p. nor oral administration.

7. In farmed fish models using rainbow trout (*O. mykiss*) juveniles, VHSV-G-frg16^{NP} delivered by i.p. injection, without adjuvant, raised specific anti-VHSV IgM, determined by ELISA. These antibodies were shown to be functional, able to reduce VHSV infectivity *in vitro*. After VHSV infection of vaccinated fish *in vivo*, there was a marked expansion of these anti-VHSV IgM, indicating immune memory was established. Survival results and viral load were consistent with protection being conferred by the nanoparticle and no apparent toxicity was observed.

8. Overall, we have shown that bacterial inclusion bodies made of antigenic viral proteins can be used *in vivo* to raise specific, functional antiviral antibodies in farmed fish, as a surrogate of protection. With a cheap and scalable production method, yielding a robust, practical product, the work here has demonstrated this approach has promise as a vaccine strategy.

SUPPLEMENTARY MATERIAL

ANNEX I

Near-infrared fluorescent protein nanoparticle iRFP^{NP}

Figure 1: FESEM image

Figure 2: Emission spectrum

Figure 3: Uptake in ZFL

ANNEX I

Near-infrared fluorescent protein nanoparticle iRFP^{NP}

The iRFP protein used is an iRFP720 (1). To produce the nanoparticle iRFP^{NP}, the plasmid iRFPHis cloned in pET22b (Genscript), was transformed into *E. coli* BL21(DE3), followed by inclusion body (IB) production as described in Chapter 1, section 3.1.2. This IB was used as a non-immune-relevant control nanoparticle. The following figures show morphology and size, emission spectrum and uptake in ZFL (zebrafish liver cell line).

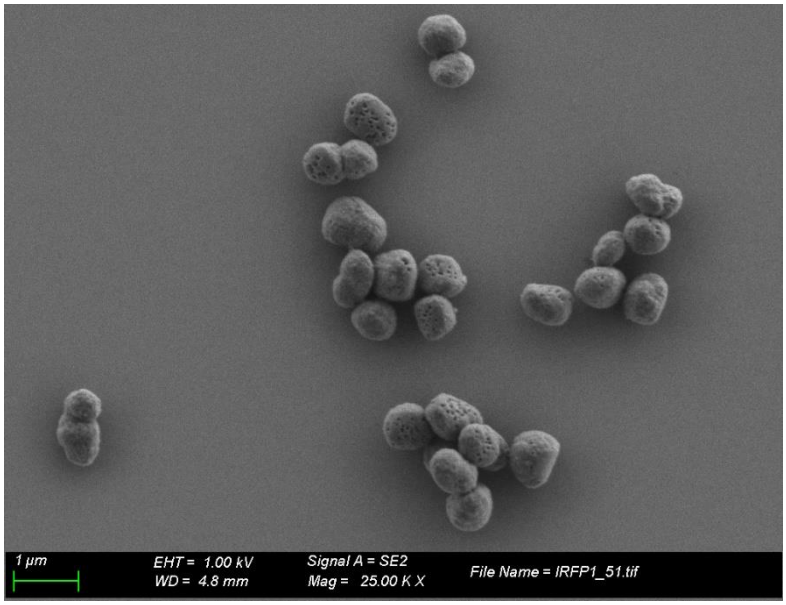

FIGURE 1: **Image of iRFP^{NP}** taken under Field Emission Scanning Electron Microscope (FESEM, Zeiss Merlin)

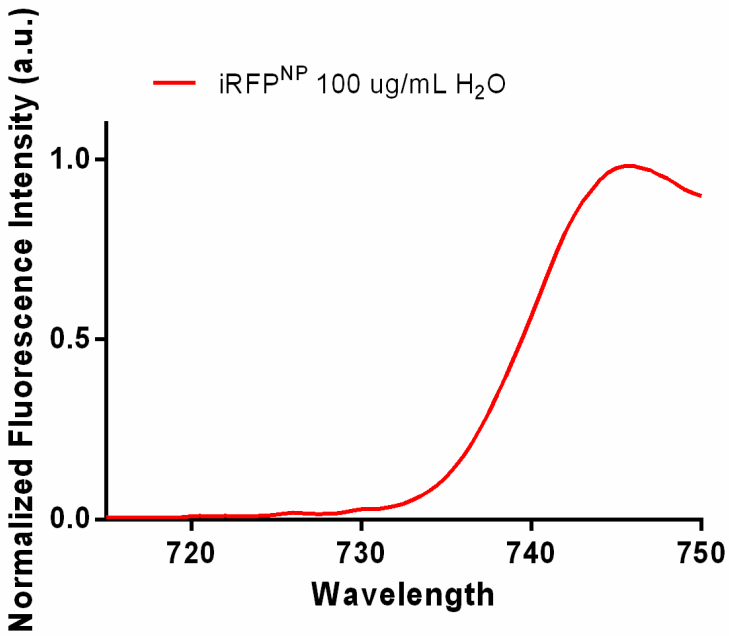

FIGURE 2: Emission spectrum of iRFP^{NP}

iRFP^{NP} at 100 µg/mL in H_2O was scanned on Jasco FP8200 fluorometer to determine excitation & emission spectra. Excitation maximum was between 695-710 nm & the emission peak was at 745 nm. The limit of detection of the instrument was 750 nm.

Note the fact that these IBs are fluorescent indicates that the functionality of the protein is maintained in the nanostructure

In previous studies, iRFP^{NP} was shown to provide protection *in vivo* against a lethal challenge with *Pseudomonas aeruginosa* when injected i.p. to zebrafish (2, 3). This was presumably due to the bacterial remnants carried by IBs which can act as immunostimulants, discussed in the General introduction of this thesis, section 6.1. However, in previous work uptake had not been explicitly demonstrated as standard cytometry equipment does not provide the possibility to excite and detect the emission of this fluorophore.

After unsuccessful attempts using a non-optimal excitation wavelength (640nm) and red and infrared filters for detection, we decided to label iRFP^{NP} with Atto-488 NHS ester (Sigma-Aldrich) and use the green channel for detection, as described for the other nanopellets (Chapter 1, section 3.1.2 and the protocol in (4)).

We wished to confirm uptake to validate using iRFP^{NP} as a control nanoparticle. A time course uptake experiment in ZFL was set up using Atto labelled iRFP^{NP} and VNNV-C^{NP} for comparison following the same protocol as described in Chapter 1, section 3.2.2. We used NPs at 5 µg/mL and measured uptake over 48 h at 3 time points. Results are shown in **Figure 3**.

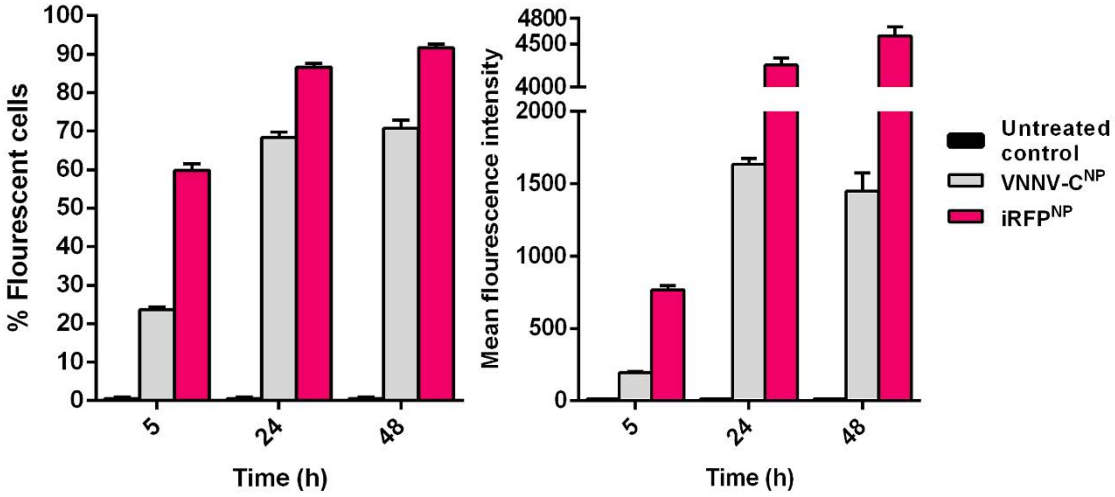

FIGURE 3: Timecourse uptake of iRFP^{NP} by ZFL. Cells were incubated with fluorescently labelled iRFP^{NP} or VNNV^{NP} at 5 µg/ mL for 5, 24 or 48 h in triplicate & uptake was analysed by flow cytometry.

Figure 3 clearly shows that iRFP^{NP} is taken up by ZFL and therefore can be used as a control nanoparticle. IRFP^{NP} is taken up more rapidly and by more cells than VNNV^{NP}, as did IPNV^{NP} and VHSV-G-frg16^{NP} (See figure 4 of Chapter 1). Here maximum % fluorescent cells (left) was ~ 90 % for iRFP^{NP} and ~ 70% for VNNV^{NP}. Interestingly, cells were still taking up iRFP^{NP} at 48 h (right) as the mean fluorescence intensity (MFI) increased slightly from 24 h to 48 h. On the contrary, VNNV^{NP} MFI had started to drop between 24 and 48 h. We observed this in our previous experiments with all the nanopellets made of antigenic viral proteins (Figure 4 of Chapter 1). Possibly the cells metabolize the viral antigens more rapidly than a non-immune relevant protein.

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ACKNOWLEDGEMENTS

I would like to thank the funding body AGAUR, Generalitat de Catalunya, for awarding me a pre-doctoral FI scholarship which has made all this work possible.

In addition, I would like to formally thank FAPESP, Sao Paulo Research Foundation, Brazil, who sponsored my participation in the Sao Paulo School of Advanced Sciences on Vaccines in November 2018. There were many inspiring high-profile researchers and clinicians involved who came together to make a great learning environment.

Then, there are many people who have helped make these three years and three months a unique and intense (!) learning experience.

In Barcelona: My supervisor Nerea Roher who took me on, believing **it is never too late to lab!** You have broadened my view of immunology from "just mammals" to a whole world of fish. Debora Torrealba who helped me so much during the initial stages of this project. Thank you for your expertise, time and friendship. Mireia Pesarrodona who was and will always be my "protein guru". Jie Ji whose curiosity and energy is alive and kicking in the lab and who helped me a lot with zebrafish. And last but not least, Joan Carles Balasch, whose talent for science and design transformed my congress posters into informative and attractive works of visual communication.

In Puerto de Santa Maria, Cádiz: Manuel Manchado and team, especially Concha Berbel and Manolo Patricio, who taught me how to manage *in vivo* experiments on a larger scale with *Solea senegalensis* and who have changed my understanding of what is "fish and chips" forever.

In Elche: Maria del Mar Ortega-Villaizán and all the team who were very supportive in the work with VHSV and introduced me to the trials, tribulations and excitement of viral challenge experiments.

At home: Finally, thanks to Chris and Rocio who never questioned their mother's decision to leave a stable, well-paid job and "do stuff" with fish, and Enrique who always understood.