Studies of Oral Vaccination against Infectious Pancreatic Necrosis in Atlantic salmon: Focus on Antigen Production, Antigen Kinetics, and Immune Responses

Studier av oral vaksinering mot infeksiøs pankreas nekrose hos atlantisk laks: Fokus på antigen produksjon, antigen kinetikk, og immunrespons

Philosophiae Doctor (PhD) Thesis

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2 Summary

Infectious pancreatic necrosis has been one of the serious diseases that cause large economic lost every year in salmonid aquaculture. Although commercial injection vaccines against IPN are used regularly in several salmon-producing countries, oral vaccines are the preferred option from a fish farmer's perspective, because oral administration is less labor-intensive, stress-free, and applicable to smaller fish. The development of oral vaccines is confronted by three challenges: (1) inefficient antigen production since huge quantities are required, (2) limited knowledge of antigen kinetics related to antigen uptake, and (3) equivocal immune responses, local and systemic, as regards humoral immune responses and immune tolerance.

In this thesis, three studies were performed to investigate those three challenges of oral vaccination. The antigen chosen for the oral vaccine was an inactivated antigen (IPNV), considered safer than attenuated virus and plasmid DNA as well as more immunogenic than subunit protein vaccines. In paper I, AGK cells showed better ability to support IPNV production compared to the other available cell lines such as RTG-2. Improved production efficacy is likely associated with low antiviral responses in AGK cells and high cell productivity. The rapid growth of AGK cells (two passages per week at 1:4 split ratio), and high viral titer (10^9 $TCID_{50}$/ml) obtained with these cells could supply large quantities of antigen for vaccine production. In the second study, the antigen kinetics including uptake, delivery, and distribution were investigated. Orally delivered, inactivated IPNV was taken up efficiently and detected in immune organs, at levels similar or higher than those of the other three groups: orally intubated live IPNV, anally intubated live IPNV, and anally intubated inactivated IPNV, suggesting that oral vaccination using inactivated antigen can be an effective strategy against IPN. Finally, immune responses following oral boost demonstrated that when inactivated IPNV is encapsulated with marine-alginate and used as a boost vaccine, local and systemic humoral immune responses were up-regulated (mRNA) such as IgT and IgM. However, it appeared that immune tolerance, not humoral immune response, was induced if only inactivated IPNV (non-formulated) was used for boost.

Overall, this thesis provides insights to the three major challenges of oral vaccine for IPNV: (1) antigen production, (2) antigen kinetics, and (3) induction of immune responses. Furthermore, the results imply that the oral alginate encapsulated inactivated vaccine would have a potential to

protect Atlantic salmon against IPN, although challenge trials are needed to confirm this in lab and in field studies.

3 Sammendrag

Infeksiøs pankreasnekrose har vært en av de alvorlige sykdommer som forårsaker store økonomiske tapt hvert år i akvakultur over de siste tiårene. Det finnes injeksjonsvaksiner mot IPN, men orale vaksiner er den foretrukne varianten sett fra et oppdretterperspektiv av den grunn at oral administrasjon er mindre arbeidskrevende, stress-fri, og kan appliseres for liten fisk (som i dag ikke kan injiseres). Utviklingen av orale vaksiner er konfrontert med tre utfordringer: (1) kostbar antigen produksjon, (2) mekanismene og kinetikken for antigen opptak fra tarmen er lite kjent, og (3) tvetydige immunresponser som inkluderer humorale immunresponser og immuntoleranse.

I denne avhandlingen ble tre studier utført for å adressere disse utfordringene. Vi valgte å studere IPN hos laks og baserte studiene på inaktiverte antigener, som er sikrere enn attenuert virus, og plasmid DNA, og gir generelt bedre immunrespons enn subenhets-proteiner (rekombinante proteiner). I artikkel I, fant vi at AGK gir bedre celler IPNV titer i forhold til andre tilgjengelige cellelinjer som RTG-2 og CHSE. AGK gir høyere titer på grunn av de lave antivirale responser og høy replikeringsrate. Rask vekst for AGK-celler (to passasjer med 1 til 4 split ratio hver uke), og et høyt virustiter (opp til 10^9 TCID50 / ml) gjør at man kan produsere store mengder antigen i løpet av relativt kort tid.. I den andre studien, ble antigen-kinetikk studert med vekt på opptak og systemisk distribusjon undersøkt. Inaktivert IPNV levert via oral rute ble tatt opp effektivt fra tarmen og påvist i immunorganer, og de nivåer som ble påvist var høyere enn for de tre andre gruppene: oral, levende IPNV, analt intubert levende IPNV, og analt intubert inaktivert IPNV. Dette tyder på at oral vaksinasjon med inaktivert antigen kan være en effektiv strategi mot IPN. I den tredje studien fant vi at immunresponsen etter oral boost med inaktivert IPNV innkapslet i marint alginat, opp-regulerte lokale og systemiske humorale immunresponser som IgT og IgM. Hvis inaktivert IPNV antigen (alene, ikke-formulert) ble det påvist immuntoleranse.

Samlet gir denne avhandlingen innsikt til de tre store utfordringene for orale vaksiner; (1) antigen produksjon, (2) antigen kinetikk, og (3) induksjon av immunresponser. Resultatene gir gode indikasjoner for at oral levert antigen, innkapslet i marint alginat har en mulighet for å beskytte laks mot IPNV smitte. Framtidige studier bør inkluderesmitteforsøk for å bekrefte beskyttelse.

4 Acronyms/Abbreviations

IPNV	-	Infectious pancreatic necrosis virus
VP	-	Variable protein
DNA	-	Deoxyribonucleic acid
ISAV	-	Infectious salmon anemia virus
SAV	-	Salmonid Alphavirus
SVCV	-	Spring viraemia of carp virus
VLP	-	Virus like particle
SVP	-	Subviral particle
MHC	-	Major histocompatibility complex
IHNV	-	Infectious hematopoietic necrosis virus
VNNV	-	Viral nervous necrosis Virus
IFN	-	Interferon
Mx	-	Interferon-induced GTP-binding protein Mx
RTG-2	-	Rainbow trout gonad cells-2
CHSE-214	-	Chinook salmon embryo 214
AGK	-	Asian grouper cell strain K
TCID	-	Tissue culture infective dose
M cell	-	microfold cell
^{3}H-LPS	-	^{3}H-labeled Lipopolysaccharide
MMC	-	Melanomacrophage center
Ig	-	Immunoglobulin
GALT	-	Gut-associated lymphoid tissue
Treg	-	Regulatory T cell
TGF-β	-	Transforming growth factor β
IL-10	-	Interleukin 10
FOXP3	-	Forkhead box P3
Th	-	T-cell helper
qRT-PCR	-	Quantitative reverse transcription polymerase chain reaction
IHC	-	Immunohistochemistry
ELISA	-	Enzyme linked immunosorbent assay
UNENCAP	-	Group given feed containing unencapsulated IPNV antigens

ENCAP	-	Group given feed containing alginate-encapsulated IPNV antigens
FBS	-	Deoxyribonucleic acid
MOI	-	Multiplicity of infection
IFAT	-	Indirect fluorescent antibody technique
RNA	-	Ribonucleic Acid
d.p.i.	-	Day post intubation
SDS-PAGE	-	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF	-	Polyvinylidene fluoride
OD	-	Optic density
CD	-	Complementary determinant
GATA-3	-	Transcription factor that recognize (A/T)GATA(A/G) motifs in DNA

5 Papers

I. Lihan Chen, Øystein Evensen, and Stephen Mutoloki. Delayed protein shut down and cytopathic changes lead to high yields of infectious pancreatic necrosis virus cultured in Asian Grouper cells. *J. Virol. Methods,* 195, 228–235, 2014

II. Lihan Chen, Øystein Evensen, and Stephen Mutoloki. IPNV antigen uptake and distribution in Atlantic salmon following oral administration (Manuscript).

III. Lihan Chen, Goran Klaric, Simon Wadsworth, Suwan Jayasinghe, Tsun-Yung Kuo, Øystein Evensen and Stephen Mutoloki. Augmentation of the antibody response of Atlantic salmon by oral administration of alginate-encapsulated IPNV antigens. PLoS One, in press.

6 Introduction

6.1 General background

Infectious pancreatic necrosis disease (IPN) is a serious problem in the aquaculture industry affecting a number of fish species [1]. It was first reported as an acute catarrhal enteritis infecting brook trout (*Salvelinus fontinalis*) in Canada in 1940 [2], and the infectious agent was isolated and named infectious pancreatic necrosis virus (IPNV) by Wolf *et al*. in 1960 [3]. Currently it has a world-wide distribution, and has been isolated from more than 32 fish species, 11 molluscs and four crustacean species [4]. The disease has been a major concern in brook trout, rainbow trout, and Atlantic salmon initially in North America and subsequently in Europe since the early 20th century [2;5;6].

The economic importance of IPN is mainly due to the mortality that it causes and the high morbidity associated with the virus [7]. In salmonids, IPNV infection causes more than 70% mortality in hatchery stocks over a period of two months [8]. Survivors of infection may become asymptomatic carriers and shed virus in their feces and reproductive products throughout the whole life [9]. From the 1990s, IPN became significant in terms of mortality and economic impact in the marine environment, when acute losses in post-smolts were reported shortly after their introduction to sea water [10-12]. The general observation was that IPN occurred 5–8 weeks after seawater transfer [13], and a decline in losses normally started after the peak summer months [14]. As the salmonid aquaculture industry has expanded extensively in recent decades, economic losses due to IPNV were estimated to be over $60 million in 1996 [15;16]. Although injectable vaccines have been developed and licensed [17], the incidence of IPNV remains high particularly in Chile while in Norway we have seen a declining trend over the last 3-4 years [10]. Moreover, these vaccines are limited by the size of fish due to the reliance on injection for vaccination.

6.2 Oral vaccines

Three major routes of IPN vaccine delivery are used for aquatic vaccination: injection (intraperitoneal and intramuscular), immersion, and oral administration. Each delivery route has its own advantages and limitations. In this thesis, oral administration was selected as the delivery method under study.

Oral vaccination is the most desirable method from a fish farmer's perspective. The advantages are stress-free vaccination with no handling of the fish, possibilities for multiple boost and the possibility for boost immunization in open water [18]. Unlike the injection delivery route, fish are not handled individually during oral delivery and therefore oral vaccination can be applied to small fish in order to reduce the cost and mortality associated with handling of the fish caused by delivery of injectable vaccine. Although immersion vaccination also offers the advantage of zero handling stress [19], it usually requires a combination of other strategies to improve efficacy, such as reduced water level, use of ultrasound, hyperosmotic infiltration, or attenuated bacterial delivery, which also stress the fish. Because of low stress, reduced labor-costs, and no restriction on fish size, oral vaccines can be used repetitively in the lifespan of a fish. Oral vaccines can be delivered to juvenile fish just developing immunity, to fish prior to release in seawater. Recently, oral administration has been shown to induce antigen-specific antibodies with immune cells in the skin mucus, bile or intestine in several fish species [20;21]. Since both intestine and skin have been suggested as possible routes of entry for IPNV and other pathogens, building local defense mechanisms involving the mucosal immune system is probably important [22]. Allnutt *et al*. (2007) suggested that fish vaccinated orally against IPN had an immune response greater than that observed in naïve and control rainbow trout after an 8 weeks induction period [23]. Therefore, vaccination via mucosal surfaces such as the intestine is an attractive delivery mode for protection against IPN [24].

Antigens delivered orally are delivered as an in-feed formulation, top-coated or incorporated into the pellet. Unlike the injection vaccine, it has to pass through the gastric juice and be exposed to digestive enzymes before reaching intestine (foregut and/or hindgut). Hindgut has been considered the prime site of antigen uptake [25]. Many novel methods have been reported to ensure the dose and quality of the antigens are maintained by the time they arrive in the hindgut. The antigen protection strategies include nano- or micro- particles and bio-encapsulation. The antigens are either encapsulated within the particles or covalently linked to the particles. Often the compounds used to form these particles are acid resistant and biodegradable. They help protect the antigens through the stomach to the intestine thus making them attractive for immunization. For example, alginates which are polysaccharides found naturally in brown algae have been used to encapsulate antigens in several oral vaccine studies. Marine alginates are ideal polymers for use in the oral vaccine delivery systems with protective features. Alginate encapsulation provides an effective method to protect bioactive compounds from acidic and

proteolytic degradation in gastrointestinal tract, because alginates shrink at low pH and dissolve at high pH. Therefore, most antigens are shielded through the stomach, and after they have passed the acidic part of the digestive tract and reach the hindgut (with pH>7), a release from the alginate matrix occurs. Joosten *et al.* (1997) were the first to employ the alginate encapsulated technology to deliver a commercial *V. anguillarum* vaccine in both carp and rainbow trout [26]. Although the vaccine was taken up by both species, the antibodies were only significantly induced in carp but protection was not measured. Romalde *et al.* (2004) successfully administered an alginate encapsulated *Lactococcus garvieae* bacterin to rainbow trout, with 50% protective level by oral immunization while the alginate-encapsulated vaccine used as booster immunization strategy showed 87% relative protection against lactococcosis. These results suggested that while the vaccine may not be suitably immunogenic itself, it may be suited to boost fish previously vaccinated by injection [27]. Recently, Leal *et al.* (2010) examined various delivery routes of a *Flavobacterium columnare* bacterin in Nile tilapia (*Oreochromis niloticus*), including formalin-killed bacteria in alginate microparticles delivered orally and bacterin delivered by intramuscular injection, intraperitoneal injection and immersion. Unfortunately, none of these delivery methods resulted in protection. Additionally, a significant humoral immune response was only detected in fish immunized by i.p. or i.m. methods [28]. In contrast, De las Heras *et al.* (2010) showed that alginate microsphere encoding DNA plasmid made from VP2 of IPNV showed high protection in immunized fish (80%) challenged 15 and 30 days after vaccine delivery [29].

Bioencapsulation has been utilized for vaccines and other materials in aquaculture such as for microalgae, artemia, and plant expressed vaccines. The microalgae *Chlamydomonas reinhardtii* was used as an oral vaccine delivery system for fish [30]. When *Renibacterium salmoninarum* protein 57 gene was transformed and expressed in the algae and given to the fish, the observation of systemic antibody response indicated the acid resistant cell wall of *Chlamydomonas* enabled the proteins to reach the posterior gut. Similarly, Companjen *et al.* (2006) delivered the transgenic potato tubers that expressed part of the *E. coli* gut adhesion molecule (LBT) fused to either green fluorescent protein (GFP) or viral peptides to carp and this resulted in an antibody response [31]. Artemia was also used to bioencapsulate antigens or rather as a vehicle. When Artemia was fed to fish following exposure to the antigen, the immune response against the pathogen was induced in older carp and gilthead sea bream [32] with 81% of vaccinated fish surviving compared to 31% of the controls post challenge in zebra fish [33].

6.3 Vaccine types

Four types of antigens are usually used for vaccination against viral diseases namely; (i) inactivated virus, (ii) recombinant viral protein (subunit), (iii) attenuated virus, and (iv) plasmid DNA. In addition comes virus-like particles (VLP), replication deficient viruses various fusion-proteins of protective antigen and an immunomodulator. Not all these technologies are applied for fish and in addition there are a limited number of reports that focus on the use of oral vaccines for fish. Therefore, the discussion here is not confined to oral vaccines, but includes other vaccine delivery systems.

6.3.1 Inactivated viral vaccine

Inactivated virus preparations are commonly used in vaccine preparations/formulations. In fish, they were the earliest type of vaccines and are still a reliable and popular strategy of immunization, and they constitute a great opportunity for studying vaccine responses in fish. Many inactivated vaccines for fish are commercially available, such as the Alpha Ject® series from PHARMAQ AS against IPNV, Micro-1 against ISA, an experimental vaccine against pancreas disease (PD), and a vaccine against spring viremia of carp (SVCV). Compared to attenuated and DNA vaccines, inactivated viral vaccine are safe for use as they are not infectious and do not cause horizontal transfer like live virus which could cause adverse ecological effects to the aquaculture environment unless well controlled. Inactivated vaccines can also induce strong immune responses since they allow use of adjuvants and also retain all exposed antigens of the virus that are important to stimulate the host immune responses. Previous studies have shown that inactivated whole viral vaccines gave better protection than recombinant subunit vaccines for IPN [34;35]. The weak points of inactivated vaccine are the short protection period if adjuvants are not included and high expense and time of antigen production. Therefore, efficient virus culturing systems are important for inactivated vaccine in addition to adjuvants.

6.3.2 Subunit viral vaccines

Subunit, protein-/peptide-based vaccines are also used to some extent. Specific viral proteins, usually capsid proteins with good immune-stimulating ability, are produced in a recombinant

system allowing rapid production of a single or small number of viral antigens. Subunit vaccines can include a non-native form of the antigen with little secondary structure and no tertiary structure formed or recombinant proteins can self-assemble and make virus like particles (VLPs) and subviral particles (SVPs) that attain the form of the virus or a smaller version. As an example of subunit IPN vaccines, VP2 and VP3 were expressed in vitro and formed VLPs [36] because of the intrinsic ability of viral structural proteins to self-assemble [37-39]. These VLPs were similar in size to the native virus and induced a strong immune response in salmon [36;40]. A more recent study described an IPN vaccine based on a SVP made from the VP2 of IPNV [23]. In this study, the subunit vaccine induced a strong immune response against IPNV [41]. Three other vaccines based on the VP2 of IPNV are already marketed under the trade names IPNV (licensed in Chile, Centrovet, Chile), Norvax (Intervet-International BV, The Netherlands), SRS/ IPNV/*Vibrio* (licensed in Canada and Chile, Microtek International Inc., British Columbia, Canada). However, previous studies are indicative of the protection of subunit vaccine being weaker than inactivated vaccine when the doses were comparable [34].

6.3.3 Attenuated viral vaccine

Attenuated viral vaccines were the first type of vaccines to be used in the history of vaccinology. These vaccines are live viruses that have been selected for non-virulence and cross reactivity (against the pathogenic strains) and/or cultured under conditions that disable viral virulence. Therefore, the virus replicates at much lower rate and are less pathogenic than the wild type virus. Attenuated viral vaccines typically induce a strong and sustained immune response against the target disease, but few commercial vaccines are based on this mode of vaccine production nowadays because of safety issues. The only commercial attenuated viral vaccine in aquaculture is found in Israel for cyprinid herpes virus 3 in koi carp [42].

6.3.4 DNA vaccine

DNA viral vaccines are among the promising vaccine preparations for easy delivery by injection or through feed using naked plasmid DNA encoding a selected gene/part of a gene of the pathogen. Upon injection using the intramuscular route, the antigen is expressed and presented via both MHC I and MHC II to lymphocytes. Therefore, a DNA vaccine can induce not only humoral immunity but also cell-mediated immunity [43]. Ballesteros *et al.* also reported that oral

DNA vaccine induced MHC II, IgM^+, IgT^+, and $CD3^+$ in the digestive tract of rainbow trout [44]. In aquaculture, the first commercial DNA vaccine was used against infectious hematopoietic necrosis virus (IHNV) in 2005 in Canada (New-Brunswick) [45]. Since then all Atlantic salmon have been vaccinated against IHN and no disease outbreaks have been seen in vaccinated populations. Under experimental conditions, DNA vaccines have been tested for their efficacy against a number of fish viruses, including Infectious Hematopoietic Necrosis Virus [46;47], Viral Nervous Necrosis Virus (VNNV) [48], Infectious Pancreatic Necrosis Virus (IPNV) [29;49] and Spring Viremia Carp Virus (SVCV) [50;51] with variable efficacy, mainly providing good protection against viruses of the family *Novirhabdoviridae.* It should be added that the production cost of these vaccines is high and there are still concerns related to the safety of the use of DNA vaccines such as induction of immune tolerance in the vaccinated animal, end-consumer safety (and GMO labeling of vaccinated fish), and the transparency/regulatory difficulties for European and many Asian markets [52].

6.4 Challenges of oral vaccines for IPN

6.4.1 Antigen production

Since it is difficult to guarantee the safety of live attenuated and DNA vaccines while inactivated vaccines show better protection than subunit vaccines, the inactivate IPNV vaccines would be the best choice for oral vaccination against IPN. It is important to produce the inactivated virus efficiently in culture systems yielding high viral concentration, especially for oral vaccines where the “consumption” of antigen is high. Previous studies suggested that a higher antigen dose of oral vaccines induced a stronger immune response than the lower antigen dose [35]. Cell culture is the best way to produce high concentrations of fish viruses so far, but cell line selection and production conditions often require optimization and the output is highly variable for the different species of virus. Therefore, a culture system that can efficiently produce high concentrations of virus is the first challenge in the production of oral vaccine.

To establish the best criteria of a good virus production system, knowledge of the viral infection process and the cellular responses post infection is necessary. Viral infection entails attachment, penetration of the cell membrane and transfer to cellular compartments, release of viral genome and initiation of transcription, protein translation, viral replication, assembly and release. Viruses

attach to the cell by viral surface antigens that bind to specific host receptors. Therefore, a cell cannot be infected without carrying a receptor for that particular virus on its outer surface. This process starts as an extracellular protein-to-protein interaction although carbohydrates and lipids are occasionally used. Once the virus gains entry into host cell, the viral genome is released and makes its way to the site of transcription, translation and replication. This can be in the cytoplasm, in organelles, or in the nucleus. Thus, the cells chosen for virus propagation should have the receptor for the virus in order to get the virus penetrate and deposit its genome in the host cell.

After entry in the host cell, the early gene products of the viral genome are translated in the cell. Most of the early proteins produced regulate the transcription of later viral genes and viral genome replication, but some of them influence the activities of host cells. For example, some translated proteins are used to induce antiviral immune responses or to control the life cycle of host cells, and subsequently to help the viral replication smoothly. For example, VP5 of IPNV serotype Ab was found to counteract the defense mechanisms that trigger apoptosis before necrosis in the host [53]. It does so by affecting the cellular gene encoding "suicide" of the early replication phase and can thus enhance the replication of the virus [53].

The later genes products are normally structural proteins required for the construction of new capsids have the viral epitopes required for the next infection. The last step is the packaging of the newly produced viral genome and virion proteins followed by the release of viral particles by budding-out, necrosis, or apoptosis. On the other hand, infected cells usually have some anti-viral reaction following virus infection. The production of interferon (IFN) by some cell types has been linked to decrease in virus replication [54]. Galligan *et al.* (2006) suggested that cellular IFN is produced by infected cells and acts on neighboring cells by inducing the production of many IFN stimulated genes including Mx to create an antiviral state [55]. In addition, apoptosis and protein shutdown are also well-known mechanisms employed by cells to protect themselves against virus infections [56-58]. These responses are also used by viruses to subvert the host antiviral responses in some cases [59;60]. Based on the infectious process described above, the following are some of the requirements needed good virus producing systems; (1) cells must provide the appropriate conditions that virus can enter the host cell, unpackage the viral genome and allow the virus to reach the area of replication, (2) cells must have a high growth rate and high confluent density that supply more resource to virus required by the virus to synthesize its own protein and genome, and (3) cells must induce a weak antivirus response following infection,

(4) the cells should also not enter into apoptosis and/or necrosis to allow sustained production and release of virus progeny and (5) facilitate release of virus from the cell.

For IPNV, although several cell lines have been tested for their suitability to propagate the virus [61] and some of them are indeed used for IPNV propagation by several scientists that include rainbow trout gonad 2 (RTG-2) and Chinook salmon embryo 214 (CHSE-214) cells, the cell culture systems are not economically viable since typical yield for IPNV Sp serotypes, which causes most outbreaks in Europe, are 10^6–10^7 pfu/ml in RTG-2 cells [62]. Further to this, RTG-2 and CHSE-24 require many days in culture before they have reached a sufficient confluence to be infected and for large volumes, these characteristics are prohibitive. Recently, a new cell line Asian Grouper strain K (AGK) has shown the capacity to grow rapidly in culture becoming confluent at with 3 days post split, and it yields a virus titer of 10^9–10^{10} $TCID_{50}$/ml depending on virus strain used [34]. Therefore, this cell-line represents a better solution for production of high concentration of IPNV antigen. These cells are derived from the skin of a crossbreed between Orange-spotted grouper (*Epinephelus coioides*) and Malabar grouper (*Epinephelus malabaricus*). The cell line is not a mono-clone but is composed of different cell types. AGK cells grow at a higher temperature, can be split at high ratios and have a much shorter turn-around time making them a very valuable resource for easy production of IPNV in large quantities within a short period of time. However, the investigation and documentation of the factors that permit the production of high titers and high volumes of IPNV are absent. Better understanding of the dynamics of IPNV infection in AGK cells will contribute to a better vaccines being used for aquaculture.

6.4.2 Antigen Kinetics

Oral vaccination is a popular topic these years and has shown good protective efficacy under experimental conditions. Although elements of the protective immune response against IPNV in Atlantic salmon have been discussed by various scientists [63-65], there is no any evidence that categorically shows that viral vaccines really passing through the gut barrier and that viral antigen reach the immune organs of fish such as head kidney and spleen. The missing of antigen uptake and delivery in these studies is a crevice of oral vaccination studies and probably accounts for the reason of the inconsistent efficacy of oral vaccines in fish farm. In mammals, the mucosal

system of the intestine is highly organized in form of Peyer's patches and lymphoid follicles [66]. The Peyer's patches possess microfold cells (M cells) that do not contain lysosomes and can therefore transport foreign antigens intact across the cells to enable an integration of innate and adaptive mechanisms [67;68]. This property has not conclusively been shown in any epithelial cells of fish. However, the existence of M-cell like cells has been proposed for salmonids [69]. For IPNV, elements of the protective immune response against IPNV in Atlantic salmon have been elucidated by various scientists [63-65] but the initial IPNV invasion of the host is still disputed. The dispute leaves a demand for understanding the uptake and organ distribution of antigens in Atlantic salmon following oral and anal administration. Several scientists have studied the uptake of various types of antigens in fish following oral and anal intubation. Dalmo *et al.* (1998) showed that the spleen and liver contained high amounts of radioactivity in turbot, but the intestine only in Atlantic cod after oral treatment with ^{3}H-LPS [70]. In rainbow trout and carp, the gastro-intestinal tract was suggested as one of the primary areas where bacterial antigens are take-up after exposure followed by presentation to central immune organs involved in the final antigen processing [65;71]. In salmonid, the second segment of the mid-intestine also represents evolutionary early antigen-sampling enterocytes [69] and the anal administration induces stronger immune response against bacteria than oral administration does [72]. Only a few studies have been concerned with IPNV uptake and organ distribution following vaccination from intestine. IPNV was detected in the cow's gut up to 72 h after feeding a silage mixture containing IPNV [22]. Both proximal and distal intestines were suggested sites of IPNV antigen uptake in Atlantic salmon [73].

Once the antigen crosses the intestine barrier, it is transported to immune organs i.e. head kidney and spleen where an antibody response is induced. The head kidney of teleost fish is equivalent to the bone marrow in other vertebrates [74]. The head kidney is composed of a network of reticular fibers that provide support for the lymphoid tissue and are found scattered among hematopoietic system cells that line the sinusoid reticuloepithelium [75]. The main immune cells are macrophages, which aggregate into melanomacrophage centers (MMCs), and lymphoid cells, which exist mostly as B cells [75]. Components of the spleen include splenic ellipsoids, MMCs and lymphoid tissue in fish. The ellipsoids are thick-walled capillaries that open in the pulp and are around the other two components. The emerging ellipsoids are involved in the capture of antigen in several species such as Atlantic salmon, grouper, catfish, and zebrafish. The antigen is engulfed by macrophage along the wall and may be detained for long periods of time, which has

an important role in immunological memory [76;77].

6.4.3 Immune response

A good oral vaccine has to induce both humoral and mucosal antibody response. After fish have been vaccinated with an inactivated antigen, naïve T-cells differentiate into effector T-helper 2 (Th2) cells. The activated Th2 cells stimulate B-cell proliferation, which induce the production of antibodies. Thus far, only three Ig isotypes have been characterized in salmonid B lymphocytes namely IgM [78;79], IgD [78;80], and IgT [81], and it is still an unclear but important topic to understand their functional roles in systemic and local immune responses following vaccination from the oral route.

IgD

IgD exists as a membrane bound Ig although it is also secreted into serum. The knowledge of IgD is still limited whether in mammal or fish. Some studies showed >90% of IgD secreting plasma cells were in the kidney and spleen in rainbow trout and the levels of IgD in the blood were generally low accounting for <2% in rainbow trout and <3% in human [82;83]. Their results also displayed IgD level correlated with increase in several viral infections [84-88] and vaccinations such as the DNA vaccine against IPNV [89]. However, this isotype was not affected by viral hemorrhagic septicemia virus infection [90]. Although Chen *et al.* (2009) suggested the secreted form of IgD bound to basophils and mast cells leading these cells to produce antimicrobial factors involved in immune defenses in mammal [83], IgD's function remains a puzzle.

IgM

IgM is the most abundant Ig isotype in serum and secreted mainly by plasmablasts and plasma-like cells that are located mostly within the head kidney, where they play a key role in eliciting IgM responses [91]. This Ig subtype plays a major role in conferring protective immunity against systemic infections. For example, Frost and Ness (1997) and Munang'andu *et al.* (2013) vaccinated fish with IPNV vaccine and reported higher levels of IgM in serum before challenge resulting in lower viral infection rate and stronger protection after challenge compared to the control fish [35;92]. Besides in the serum, IgM has been detected on mucosal surface of the intestine, skin, and gills [93-95]. Recently, Ballesteros *et al.* (2013) observed that the membrane

form of IgM was highly expressed in the pyloric caeca of fish that were vaccinated with a DNA vaccine encapsulated in alginate beads than fish exposed to empty plasmids or alginate beads alone, and they also suggested that IgM secreting B-cell maturation correlate with increase in antigen levels expressed on mucosal surfaces of orally vaccinated fish [89]. Some studies also demonstrated that IgM plays a role in mucosal response, because of the presence of antigen-specific IgM albeit with low level. However, the protective mechanism at viral entry sites has not been studied in detail.

IgT

IgT is the only known member of the gut-local immune response in fish possibly mirroring the function of IgA in mammalia. Zhang *et al.* (2010) detected responses of rainbow trout immunoglobulin T (IgT) to an intestinal parasite only in the gut, whereas IgM responses were confined to the serum [95]. Further studies indicated that this isotype was mainly involved in mucosal immune responses both in the gut and skin [95;96], and thus its titer in mucus and mucosal surfaces are estimated to be >100 fold higher than those detected in serum [93]. IgT also was suggested to have a role comparable to IgA that acts as a mucosal antibody and had specialization into mucosal responses [97]. Moreover, IgT^+ B cells could be observed in the lamina propria and epithelium of the gut, suggesting a role of these cells in producing Ig locally [95]. Local production of immunoglobulins in the GALT probably occurs in other species as a number of studies have found B cells in the GALT of fish [98]. However, only few studies have examined the anti-viral properties of IgT in the gut post oral vaccination, such as the studies of regulation of IgT in the gut post oral or immersion viral vaccination by Cain *et al.* (1996) and Sato and Okamoto (2010) [99;100]. For IPNV, the IgT secreting cells were reported to migrate toward the peritoneal epithelial surface after IPNV exposure [101], and high level of IgT was expressed in the gut of Atlantic salmon following IPNV oral DNA vaccination [89;102]. In more recent study, Ballesteros *et al.* (2013) have shown a high correlation between the levels of VP2 transcribed from an oral DNA vaccine and the levels of IgT in the pyloric caeca suggesting that the expression pattern of this Ig isotype could be highly influenced by the quantity of antigen expressed on mucosal surfaces of vaccinated fish. Although there is no hard evidence of antiviral properties of this isotype, IgT is conceivable that it plays a role protecting surfaces against pathogens that infect through mucosal surfaces [89] like IPNV.

6.4.4 Immune tolerance

The intestine is the place where immune tolerance plays a role in avoiding food allergy and to maintain the gut microbiota. Therefore, oral immune suppression is another important coordinate element that influences oral vaccine efficiency, but few studies have been done in fish. This phenomenon has been shown to decrease specific antibody production against antigens in rainbow trout [9], salmon [103] and common carp [103-105]. Only in common carp, the oral administration of proteins could result in a genetic dependent suppression of antibody responses after systemic administration of the same antigen [105], which was comparable with oral tolerance studies in mammals. In mammals, the regulatory T cells (Treg) cells play essential roles in immune tolerance [106] (Fig 1). Treg distinctively expresses the FOXP3 transcription factor, in addition to producing inhibitory cytokines TGF-β and/or IL-10. Whether these subsets exist in fish is unknown, but many associated molecular homologies have been identified in teleost fish [107]. FOXP3 which is a member of the forkhead box (FOX) gene family is currently the most intensively studied forkhead family member in immunological research and such studies show that FOXP3 is a major regulator of development and suppressive function of Tregs in mammal[108]. Quintana *et al.* (2010) reported FOXP3 showed similar immune-regulation in zebrafish to mammalian [109], and Yang *et al.* (2012) also suggested the FOXP3 probably had the similar function in grass carp [110]. TGF-β and IL-10 are the well-known cytokines as main factors in the control of the oral immune tolerance in mammal [111]. The anti-inflammatory cytokine IL-10 and the Th3-associated anti-inflammatory cytokine TGF-β have been identified in several species of teleost fish [112-123]. Although their functions in Atlantic salmon are not clearly understood, more and more studies suggest that TGF-β and IL-10 modulate pro-inflammatory cytokines and T and B cells proliferation in goldfish, grass carp, rainbow trout and Atlantic salmon as well [123-127].

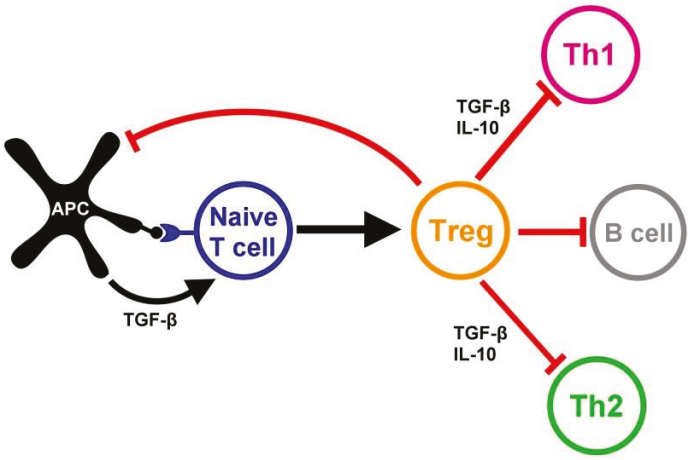


Figure 1. Treg cell development and suppression

7 Objectives

The overall aim of this work was to optimize IPNV production for use in inactivated vaccine preparations on one side and to gain knowledge of antigen uptake as well as host response following oral vaccination against IPN in Atlantic salmon. Specifically, the following objectives were examined;

- To investigate and document the factors that permit reproducible high titers and volumes of IPNV in AGK cells. A good understanding of these factors will contribute to more efficient production of antigens that are required in the development of protective vaccines against IPN.

- To determine the antigen uptake and organ distribution of inactivated vaccines given orally using different administration routes in Atlantic salmon and assess the potential of systemic distribution of inactivated IPNV antigens.

- To determinate the immune response including the systemic and local antibody response of an oral IPN vaccine used as a boost in Atlantic salmon immunized with a prime injection vaccine.

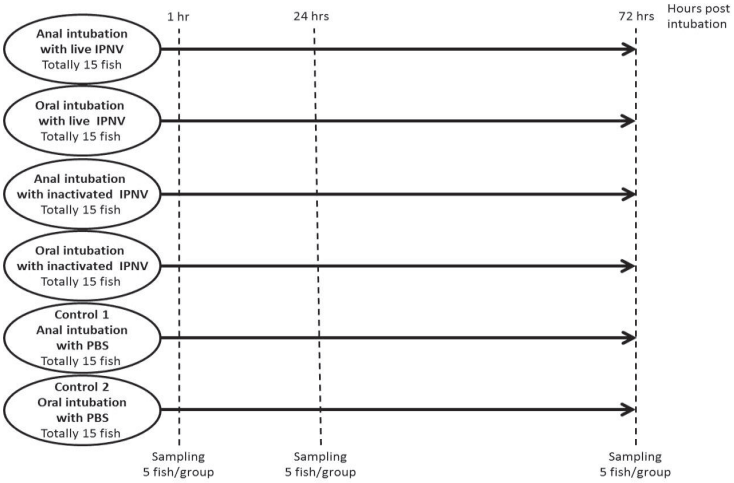
8 Methodology

8.1 Overview

For investigating the oral vaccine for IPN, the steps of research in this thesis followed the stream of oral vaccine process to vaccination. The knowledge gaps of antigen production, antigen kinetics, and immune response were explored by three studies. For the vaccine especially administrated by oral route, efficient production of antigen with high titer is desired. AGK cell line was chosen to produce for paper II and paper III, due to its property of generating high titers of IPNV and ability to proliferate rapidly. AGK and RTG-2 cells were used to grow IPNV as a basis for understanding the mechanisms underlying differences in yields. CHSE-214 cells were used to quantify virus yields from both cell lines in all trials. The stream of IPNV titer change and several anti-viral activities were detected from genome level to protein level.

Although many viral vaccines were reported to give good protection in laboratory trials, the efficacy of vaccines under field conditions is still equivocal. For oral vaccines there are very few studies documenting the efficacy and also with little information provided as regards details of antigen uptake, antigen delivery, and local adaptive immune response. The results of antigen kinetics in paper II and systemic and local immune response in paper III can surmount the gaps and provide an approach for the development of an oral vaccine against IPN. For papers II and III, the vaccines made of the AGK produced IPNV were given to the Atlantic salmon. In paper II, we studied the administration of 0.3 ml of live and inactivated IPNV (10^9 $TCID_{50}$/ml) was intubated into stomach and hindgut. The antigen was detected by qRT-PCR, immunohistochemistry (IHC), and ELISA at 1, 24, 72 hrs post vaccination in serum, foregut, hindgut, head kidney, spleen, and liver (Fig. 2A). In paper III, the boosts included: 1) untreated feed (control); 2) feed containing unencapsulated IPNV (in suspension) (UNECAP); 3) feed containing alginate-encapsulated IPNV antigens (ENCAP). The boost vaccines were were given every day for 7 days. The antigen was detected by qRT-PCR in head kidney and hindgut, and immune response was detected by ELISA and qRT-PCR at 7 weeks post the first boost and at 4 weeks post the second boost in serum, head kidney, spleen, and hindgut (Fig. 2B).

A.



B.

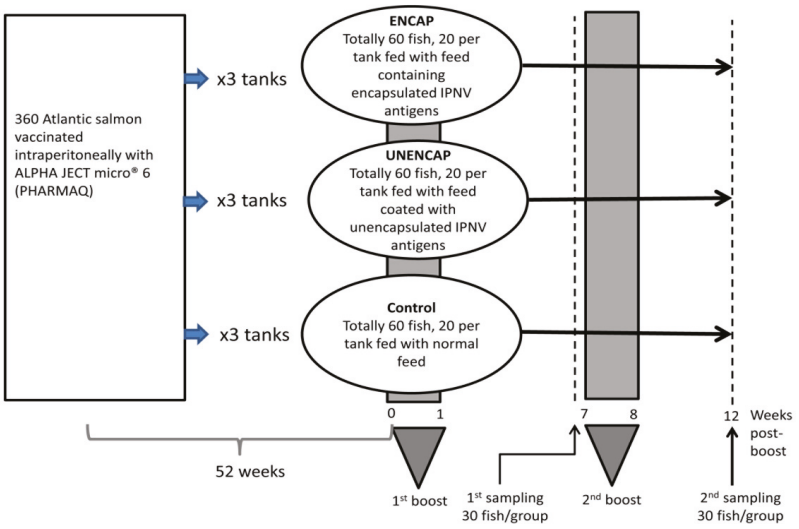


Figure 2. The design of studies: A) Paper II; B) Paper III

8.2 Cells, virus, vaccines, and fish

Rainbow trout gonad 2 cells (RTG-2), Asian Grouper strain K (AGK) cells [34], Chinook salmon embryo cells (CHSE-214) were used in this thesis. RTG-1 and CHSE-214 are widely used in IPNV studies and AGK can produce IPNV efficiently.

A recombinant IPNV strain (rNVI-15PT) having amino acids Proline (P), Threonine (T) and Alanine (A) in positions 217, 221 and 247 in the VP2 protein, respectively [35], was used to infect 80% confluent cells. This recombinant IPNV strain replicates as fast as the wild type IPNV ($T_{217}A_{221}T_{247}$) but has lower mutation rate than the wild type IPNV in cells. The AGK and RTG-2 cells were infected in triplicates by first adsorbing the virus for 2 hrs on a rocking board. Thereafter, the infected monolayer cells were washed using phosphate buffered saline (PBS) before replacing the old growth media with maintenance media followed by incubation at 15°C. AGK cells maintained using 2% FBS of L-15 growth media were inoculated with IPNV using MOI=0.1 followed by incubation at 15° C to produce IPNV..Virus samples from both intact cells and culture supernatants were harvested in parallel, on daily basis until full CPE. The virus yield in these samples was assessed by titration in CHSE-214 cells. The virus for inactivated vaccine was incubated with formalin (0.5%) at room temperature for 48 hrs with continuous stirring using a magnetic stirrer. Thereafter formalin was removed by dialysis against PBS. Inactivation was confirmed by inoculating confluent CHSE-214 cells while formalin residual effects were tested by incubating cells with excessive inactivated virus and assessing for toxicity. Inactivated antigen encapsulated by marine alginate and inactivate antigen with PBS were mixed with other feed components to make the oral vaccine in paper III. Once the antigen was ready, a trial was carried out in which Atlantic salmon parr (n = 90) approx. 25 g and (n=180) approx. 200 g were used in the studies reported in paper II and paper III, respectively.

8.3 Virus detection and quantification

Several techniques were used to detect and quantify the amount of IPNV such the Kärber's method of determining the tissue culture infective dose ($TCID_{50}$/ml), qRT-PCR, western blotting, ELISA, IFAT, and IHC. Kärber's method is used to determine $TCID_{50}$/ml. This is a method with good sensitivity for infectious virus the titer is determined by the number of wells having cytopathic effect (CPE). The $TCID_{50}$/ml titration method is time consuming and requires one week to be completed (after inoculation). qRT-PCR and western blotting were also used to quantify the virus used for studies in paper I. Those techniques have good sensitivity and resolution and require less time. qRT-PCR detection will not estimate the number of replicating virus particles. qRT-PCR was used for determining the presence of IPNV antigen in head kidney and hindgut samples in paper III. The indirect enzyme linked immunosorbent assay (ELISA) is a tool that can be used to quantify virus at protein level especially in serum because of its ease of

use, although sensitivity is moderate (≈10^4 virus particles per ml). ELISA was used for studies in paper II. Besides quantification, it was important to visualize the distribution of IPNV in internal organs and for this purpose immunohistochemistry (IHC) was used.

8.4 Gene expression

Gene expression analysis can provide insight into how the organism responds to infection and vaccination. Genes are up-regulated or down-regulated in response to different antigen or vaccine components. By quantifying mRNA levels of transcripts like cytokines, immunoglobulin and immune regulatory molecules, a transcript profile can be generated [128-132]. In this way, immune gene expression can be used to provide a fingerprint of a protective response. In the thesis, qRT-PCR was used to track and quantify several immune genes for the purpose of understanding the immune response following vaccination for studies reported in paper III. This can be used and extended in future studies including with the purpose to create correlates of protection.

8.5 Cellular antiviral activity test

Cellular antiviral responses are parameters that influence the efficiency of virus production (at cell level). Protein synthesis shutdown, antiviral ability of interferon pathway cascades and apoptosis are believed to be important antiviral responses induced by cells in response to infection. For quantification of protein synthesis, ^{35S}methionine was incubated with the cells for one hour to label the new proteins synthesized during incubation. After the protein is isolated from the cells, separated, blotted onto the membrane, and scanned by an imager, level of protein synthesis can be assessed. While the IFN sequence of grouper is still not determined, the antiviral responses induced through IFN pathways was approached by using poly (I:C) for induction of IFN. Also overexpression of Mx protein and its effect on IPNV growth/replication was also studied (Paper I). Further, apoptosis responses in infected cells were also examined (Paper I) using flow cytometry to visualize Annexin V positive cells (a marker of apoptosis).

8.6 Humoral Antibody quantification

Sandwich ELISA was used to assess antibody levels obtained at different time points in paper III. Compared to indirect ELISA, sandwich ELISA is more sensitive and with higher specificity. Since the sandwich ELISA quantifies proteins between two layers of antibodies, the protein is

specifically captured and detected. The performance followed a previous description [133] with minor modifications. An IPNV concentration of 10^7 $TCID_{50}$/well was used (as bait) while serum samples were diluted at 1:40 in phosphate buffered saline.

9 Summary of separate papers

Paper I: Delayed protein shut down and cytopathic changes lead to high yields of infectious pancreatic necrosis virus cultured in Asian Grouper cells

Inactivated whole virus vaccines represent the majority of commercial preparations used to prevent against infectious pancreatic necrosis (IPN) in salmonids today. The production of these vaccines requires high virus concentrations and is resource-demanding.

In this study, we describe the cultivation of high yields of IPN virus in Asian Grouper strain K (AGK) cells. The mechanism by which this is achieved was investigated by comparison with commonly used salmonid cell lines (RTG-2 and CHSE-214 cells). The cells were counted before and sequentially after infection. Thereafter, protein shut down, virus yields and apoptosis were assessed. The effects of poly(I:C) pre-treatment and Mx expression on IPNV concentrations were examined and the results show that high virus yields were associated with high cell numbers per unit volume, delayed cell death and apoptosis in AGK cells while the opposite was observed in RTG-2 cells. Poly(I:C) treatment and Mx expression resulted in a dose-dependent inhibition of virus multiplication. The production capacity of AGK and CHSE-214 cells were compared and higher split ratio and shorter split interval of AGK cells documents dramatic differences in virus antigen production capacity.

Collectively, the results suggest that high cell numbers and prolonged survival of AGK cells are responsible for the superior virus yields over RTG-2 and higher split ratio/shorter split interval makes AGK superior over CHSE cells.

Paper II: IPNV antigen uptake and distribution in Atlantic salmon following oral administration

One of the difficulties facing the success of oral vaccination in fish is the hostile stomach environment that antigens must cross. Further, uptake of antigen from the gut to systemic distribution is required for induction of systemic immunity, the dynamics of which are not well understood. In the present study, groups of Atlantic salmon parr were intubated with live or inactivated IPNV either orally or anally. At 1, 24 and 72hrs post infection (p.i.) the fish were

sacrificed and serum, head kidney, spleen, liver, anterior and posterior intestinal tissues were sampled. The serum was used for assessing IPNV by ELISA while tissues were used for antigen distribution by immunohistochemistry. Both live and inactivated IPNV antigens were observed in enterocytes of the intestines and in immune cells of the head kidneys and spleens of all groups. In the liver, no antigens were observed in any of the groups. Significantly higher serum antigen OD values ($p<0.04$) were observed in orally compared to anally intubated fish. By contrast, no difference ($p=0.05$) was observed in tissue antigens between these groups by immunohistochemistry. No significant difference ($p=0.05$) was observed in serum antigens between groups intubated with live and inactivated IPNV while in tissues, significantly more antigens ($p<0.03$) were observe in the latter compared to the former. These findings demonstrate that both live and inactivated IPNV is taken up by enterocytes in the intestines of Atlantic salmon likely by receptor mediated mechanisms akin to those of HRP or ferritin. Higher IPNV uptake by the oral compared to anal route suggests that both the anterior and posterior intestines are important for the uptake of the virus and that IPNV is resistant to gastric degradation of the Atlantic salmon stomach.

Paper III: Augmentation of the antibody response of Atlantic salmon by oral administration of alginate-encapsulated IPNV antigens

Atlantic salmon (*Salmo salar* L.) growers often face infectious pancreatic necrosis virus (IPNV) challenge during the first few months following transfer to sea. To be protected, fish needs high antibody titers at the start of the challenge and it is questionable whether sufficient antibody titers are induced by commercially available injection vaccines. Thus boosting with oral vaccines would be a good alternative. The effect of orally boosting fish with IPNV antigens however remains poorly understood.

The objective of the present study was to assess if alginate-encapsulated whole IPNV antigens would stimulate a booster response of Atlantic salmon post-smolt. One year after intraperitoneal vaccination with an oil-adjuvant vaccine, the fish (growers) were boosted either by 1) alginate-encapsulated IPNV antigens (ENCAP); 2) soluble antigens (UNENCAP) or 3) no antigens in feed (control). This was done twice in seven weeks intervals. The first sampling was done 7 weeks post the 1st oral boost (just before the second oral boost) while the second sampling was at 4 weeks after the 2nd oral boost. Samples collected were serum, head kidney, spleen and

hindgut. Antibodies in the serum were analyzed by ELISA while the remaining tissues were used to assess the expression of IgM, IgT, CD4, GATA3, FOXP3, TGF-β and IL-10 genes by qRT-PCR. Compared to controls, fish fed with ENCAP had a significant increase ($p<0.04$) in serum antibodies following the 1st boost but no difference was observed after the 2nd boost. This coincided with significant up-regulation of CD4 and GATA3 genes. In contrast, serum antibodies in the UNENCAP group decreased both after the 1st and 2nd oral boosts. In the second samples, this was associated with significant up-regulation of FOXP3, TGF-β and IL-10 genes suggesting the induction of tolerance. The expression of IgT was not induced in the hindgut after the 1st oral boost but was significantly up-regulated following the 2nd one. CD4 and GATA3 gene expression exhibited a similar pattern to IgT in the hindgut. IgM mRNA expression on the other hand was not differentially regulated at any of the times examined.

Our findings suggest that 1) Parenteral prime with oil-adjuvant vaccines followed by oral boost with ENCAP results in augmentation of the systemic immune response; 2) Symmetrical prime and boost (mucosal) with ENCAP results in augmentation of mucosal immune response and 3) Symmetrical priming and boosting (mucosal) with soluble antigens results in the induction of systemic immune tolerance.

10 Results and Discussion

10.1 Overview

This thesis is composed of three studies. Each focuses on challenges of the oral vaccine preparation and use, including antigen production (paper I), antigen kinetics (paper II), and immune responses (paper III). The sequence of these three studies was performed according to the process of vaccine development. Paper II and paper III would not have been done without the high titer/ high production capacity obtained with AGK cells, i.e. produced by the optimized production system reported in paper I. The results of paper II gave evidence to support that the immune responses observed in paper III were induced by IPNV antigen locally or systemically. Therefore, the findings of this thesis can make contributions to improve the development of IPNV oral vaccines for Atlantic salmon.

10.2 High yield of infectious pancreatic necrosis virus produced by Asian grouper cell strain K (AGK)

10.2.1 The effects at cell population level

AGK cells produced IPNV titers in excess of 10^9 $TCID_{50}$/ml which is higher than what is obtained for RTG-2 cells and comparable to or above previous records of CHSE-214 and CHH-1 [61;62]. Moreover, the higher split ratio and short interval of AGK cells compared to RTG-2, CHH-1, and CHSE-214 gave a very marked difference in production capacity. The number of AGK cells was more than twice of RTG-2 per unit volume at confluence. This means that AGK supply more “factories” to produce IPNV. AGK cells grow more rapidly compared to salmonid cell lines and are split 2 times a week at 1:4, very different from RTG-2, CHSE-214, and CHH-1 cells (Fig. 3). The effect of higher split ratio and the shorter split interval results in a very high number of flasks available for inoculation and the resulting vaccine volume will differ dramatically. This is a great advantage for vaccine production.

10.2.2 The effects at individual cell level

Although AGK cells have higher cell numbers per unit area than RTG-2 cells, the 100 times higher titer of AGK-produced IPNV suggested that the difference in cell numbers could only account for part of the difference in titer. The production capacity per cells is also important.

Protein shutdown and apoptosis are among the mechanisms cells use for protection against virus infections [56-58;134] but have not been clearly elucidated in IPNV infected cells. In paper I, the delay of protein shutdown (Fig. 4) and delayed/lack of apoptosis in AGK cells was associated with high host cell numbers and virus yields. This is in contrast to the findings of RTG-2 cells where cell multiplication ceased at 1 d.p.i. (MOI = 10) or 2 d.p.i. (MOI = 0.1), coinciding with host cell protein shutdown. Furthermore, apoptosis was detected in RTG-2 cells by 1 d.p.i. unlike in AGK cells where it was absent at this time point. Collectively, these findings suggest that delayed protein shutdown and CPE in AGK cells allows longer replication windows that in turn increase virus yields, opposite to what is seen in RTG-2 cells. The similarities in the trends of protein shutdown of AGK cells infected with different MOI in this study were intriguing given that the final virus concentrations were different. In AGK cells, the virus was inhibited when Mx protein was over-expressed (Fig. 5). Moreover, the induction of Mx was observed when the cells were infected with high MOI leading to low viral titers, but it was absent when cells were inoculated with low MOI, ultimately with higher virus titers. These findings support previous reports that the effect of Mx on IPNV is dose-dependent, the more the expression, the more its antiviral effects [135]. Low MOI allows the virus to multiply without inducing Mx (virus remains "under the radar") and thus results in high virus yields. The Mx protein inducer, IFN, was also reported to decrease the proliferation of IPNV. For example, Dobos and Roberts (1983) reported that the induction of IFN in RTG-2 resulted in low virus concentrations while the absence in CHSE-214 cells was linked to opposite [54]. We used poly(I:C), a potent inducer of interferon, to stimulate the AGK cells to release IFN because the IFN gene sequence of AGK cells was still unknown (at the time these studies were carried out). While no difference between poly(I:C) treated and untreated groups was observed in RTG-2 cells, poly(I:C) transfection initially inhibited virus replication in AGK cells. These results indicate lower antiviral responses induced and higher virus yield obtained in AGK cells. Moreover, this study clearly shows the potential of the AGK cell line used for production of IPN vaccine antigen.

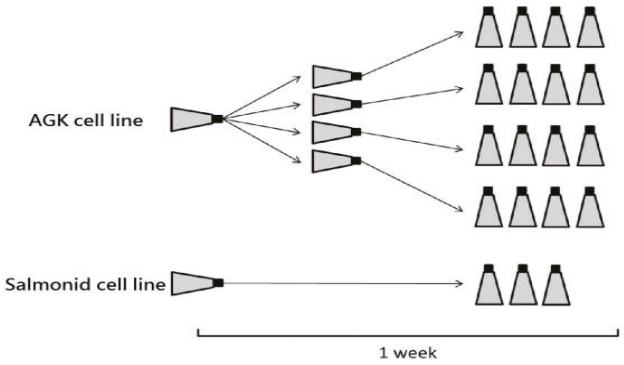


Figure 3. The spilt ratio of AGK and Salmonid cell lines (RTG-2 and CHSE-214)

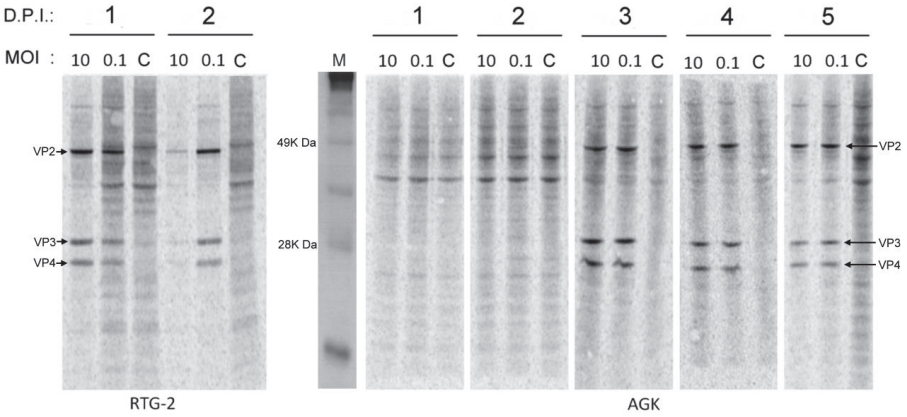


Figure 4. Protein shutdown in RTG-2 and AGK cells following infection with infectious pancreatic necrosis virus. Key: VP2, VP3, VP4 = virus proteins; M=marker; d.p.i. = days post infection; MOI= multiplicity of infection, C = negative control. Newly synthesized proteins were labelled with [35S]Methionine, separated by SDS-PAGE, blotted on PVDF membrane and imaged using a Typhoon imager.

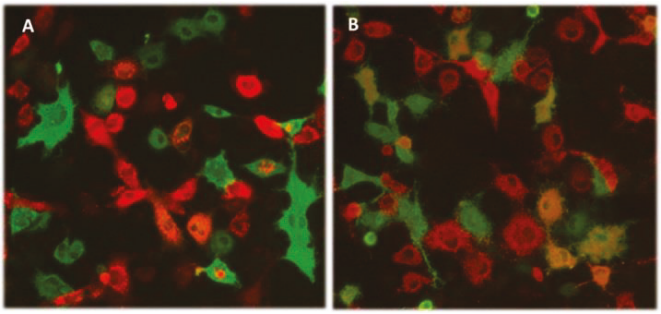


Figure 5. Indirect fluorescence antibody test of overexpressed Mx (A) in AGK cells following infection with infectious pancreatic necrosis virus at 72 h post infection. (B) Negative control transfected with the truncated form of Mx, note the degree of double staining. Fewer cells present the double staining when the cells over expressed Mx (A). Key: green color = Mx; red stain = IPNV; yellow/orange = double staining of IPNV and Mx (A) or its truncated form (B).

10.3 The IPNV antigen uptake kinetics

An ideal vaccine should mimic the natural route of infection and provide enough virus-specific epitopes to induce protective immune responses [136;137]. Therefore, understanding antigen uptake is a first step for developing a good mucosal vaccine. Since the intestine has been suggested to be one of the entry sites of IPNV [22], administrating the oral vaccine via the intestine mucosal surface would be a suitable option of vaccination. Our result shows that the uptake of this virus was from the lumen of the posterior intestines (Fig. 6A) and supports previous reports that the posterior intestine or hindgut is important for absorption of macromolecules in fish [104]. In order to understand the efficiency of IPNV uptake, indirect ELISA was used to detect IPNV antigens in serum. The OD values of all test groups were higher than control group at every sampling time point and significantly higher in anal live, oral live and oral inactivated IPNV groups at 24 h.p.i. and in oral live and inactivated IPNV groups at 72 h.p.i. (Fig. 7). The group intubated orally had comparatively more antigen than anally (by ELISA or IHC), in contrast to the findings reported previously [104;138]. Since IPNV is known to be resistant to chemical and even thermal treatments [139-141], it is not surprising that our results suggest that IPNV is resistant to the low pH and digestive enzymes in Atlantic salmon stomach. We did not observe an obvious difference between live and inactivated IPNV antigen after oral administration. These finding are consistent with previous reports indicating that formalin inactivation of IPNV does not significantly alter the surface structure of the virus [142;143].

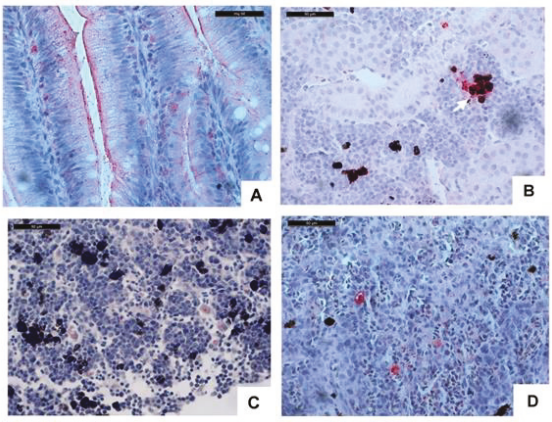


Figure 6. Infectious pancreatic necrosis virus (IPNV) in intestine (A); MMC (arrow) (B); head kidney (C); spleen (D) demonstrated by immunohistochemistry.

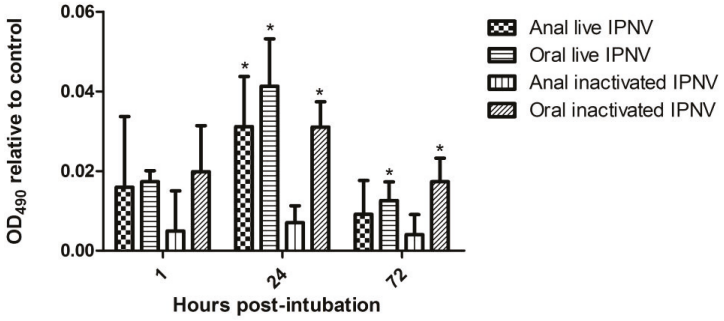


Figure 7. The relative OD_{490} values of IPNV antigen in serum of live IPNV intubated from anal route (Anal live IPNV), live IPNV intubated from oral route (Oral live IPNV), inactivated IPNV intubated from anal route (Anal inactivated IPNV), and inactivated IPNV intubated from oral route (Oral inactivated IPNV) groups to control group at 1, 24, and 72 hours post intubation. Bars represent mean values of OD + SD values from 2 to 5 individuals. *Depicts statistical significance between boosted fish versus non-boosted control ($p < 0.05$).

10.4 IPNV antigen distribution in the organs

The number of fish per group in which antigens were detected is shown in Table 1. No antigens were detected from the anterior intestine in any of the groups. Although no antigens were demonstrated in the anterior intestine in the present study, the higher serum antigens in orally intubated groups compared to their anal counterparts as assessed by ELISA suggest that the anterior intestine as well as the foregut may be important in the uptake of IPNV as suggested by some [104] while contrasting others [104;138]. In the hindgut, antigen was observed on mucosal surfaces and in macrophages and goblet cells (Fig. 6A). As far as we know, this is the first report

to document the uptake of this virus both live and inactivated from the lumen of the intestines. The anticipation is that the mechanisms involved are similar to that of HRP or ferritin as reported by others [138]. These findings support previous reports that the intestine is important for absorption of macromolecules in fish [104].

Antigens of both live and inactivated virus intubated either orally or anally were observed in the immune organs (head kidney and spleen) of the fish from 1 hr to 72 hrs p.i. Antigens were localized mostly in macrophage-like cells and melanomacrophage centers (MMCs) (Fig. 6B, C, D). To our knowledge, the MMCs were shown for the first time to be at the same location of IPNV positive stain by IHC. MMCs play an important role in the response of fish to pathogen. Ellis & de Sousa [149] showed that circulating small lymphocytes also migrated to the centers and suggested they may be the location where trapped antigen interact with the immune system [144]. Furthermore, the close relationship of MMCs with the lymphoid cells indicate they were the major sites of long-term antigen retention in fish [145]. Therefore, the IPNV and MMCs in the same location implied that live and inactivated IPNV antigen could be recognized by the immune system of Atlantic salmon parr whether intubated orally or or anally. Different from immune organs, no IPNV antigens were observed in the liver of any groups in the present study. Since salmonid liver receives mostly venous blood from the gut [146-148] and plays a role in the digestion and removal of toxins from the blood, all antigen taken up by the intestine would pass through and potentially be taken up in the liver.

Table 1. Number of fish with positive staining for infectious pancreatic necrosis virus antigens in different tissues following intubation.

Tissue	Time	Live virus		Inactivated virus	
	(hours p.i.)	Anal intubation	Oral intubation	Anal intubation	Oral intubation
Posterior intestine	1	0*	0**	1	1
	24	0	0	0	0
	72	0	0	1	1
Liver	1	0*	0**	0	0
	24	0	0	0	0
	72	0	0	0	0
Head kidney	1	3*	0**	2	3
	24	0	1	2	0
	72	0	3	3	0
Spleen	1	2*	0**	1	2
	24	0	0	3	1
	72	0	0	2	0

*n=3; **n=2 otherwise n=5.

10.5 The immune responses following oral vaccination with IPNV in Atlantic salmon

10.5.1 The systemic humoral responses

The findings of paper III demonstrate that oral boosting of injection vaccinated Atlantic salmon with alginate encapsulated IPNV antigens induces systemic humoral responses. The induction of higher transcript levels of T-helper 2 (Th2) mRNA in the ENCAP group is likely since there was a significant up-regulation of CD4 and GATA-3 in the head kidney and spleen (Fig. 8) of these fish, and IPNV-specific antibody in the serum. Induction of Th2 response was reported as one of the major immune responses from vaccination using inactivated virus and alginate encapsulated antigens in previous studies [149-152]. These findings are consistent with other reports in which different antigens were used [153;154] and suggest that oral boosting with alginate encapsulated antigens holds promise as a means of augmenting immune responses against IPNV.

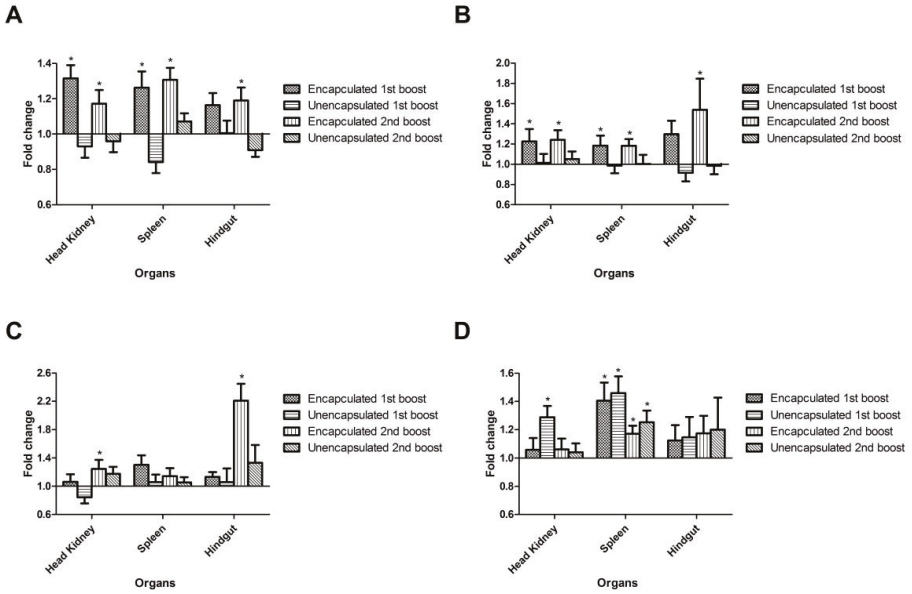


Figure 8. Mean relative expression of A) CD4; B) GATA3; C) IgT; and D) IgM genes of fish orally boosted with different antigen preparations compared to un-boosted controls. n=30; *statistically significant $p< 0.05$.

10.6 The local humoral responses

In the present study, the up-regulation of IgT was only observed in the head kidney and the hindgut at the second sampling. IgT is the only known Ig in the gut of fish that resembles the function of IgA in mammalia. However, only few studies have examined the anti-virus properties of IgT in the gut of fish after oral vaccination [99;100;155]. Since IgT is a locally induced antibody response expected to play a role in mucosal immunity, it was up-regulated in head kidney and hindgut, as expected, with the highest levels found in the hindgut (Fig. 8).

Additionally, the results in paper III show that the 1st oral boost did not induce a corresponding change in the mucosal response as measured by gene expression in the hindgut, which suggests that injection vaccination does not activate mucosal immunity, in common with findings of others [154]. Therefore, the first oral boost served as a "prime" to the mucosal response while the 2nd exposure "boosted" it. Interestingly, oral boosting did not result in increased IgM expression in the hindgut, different from what others have observed assessing mucosal antibodies [154]. The ability of orally administered antigens to stimulate both systemic and mucosal immune responses

while parenteral delivery of antigens only inducing a systemic immune response demonstrate the asymmetry seen also for other animal species (mice) [156].

10.7 Immune tolerance

Immune tolerance is one of the challenges of oral vaccination. Soluble antigens have been found to induce tolerance in carp [104]. In the present study, tolerance was induced by two consecutive administrations of unencapsulated IPNV antigen (UNENCAP). The suppression of antibodies aligns well with induction of FOXP3, TGF-β and IL-10. The expressions of FOXP3, a marker of Treg was only slightly different between three groups at first sampling, but was significantly higher in the unencapsultaed group a second sampling in head kidney and the hindgut when compared to the other group. In addition, TGF-β and IL-10 showed a similar pattern, but they were significantly up-regulated in both the unencapsulated and encapsulated groups at the second sampling (Fig. 9). TGF-β and IL-10 have been reported to be immunosuppressive cytokines linked to immune tolerance [157-159]. In mammals, TGF-β was secreted by dendritic cells (at low dose) and macrophage (at high dose) to modulate Treg, followed by reducing the activity of Th1 and Th2 cells but inducing gut associated immunoglobulin IgA secretion [111;160;161] while IL-10 was secreted by Treg to suppress the Th1 and Th2 cells and increase IgA production [111]. Induction of tolerance suggests that most of the un-encapsulated IPNV antigens were taken up, meaning they survived the hostile acidic environment in the stomach. This finding underlines the importance of encapsulation as an aid to stimulating the immune response of fish. It is noteworthy that the doses of vaccines administered during the 2nd boost were lower per body weight of fish compared to the 1st boost since the fish had gained weight by the time they received the second boost. The effect of this was not addressed but should be a subject of future studies.

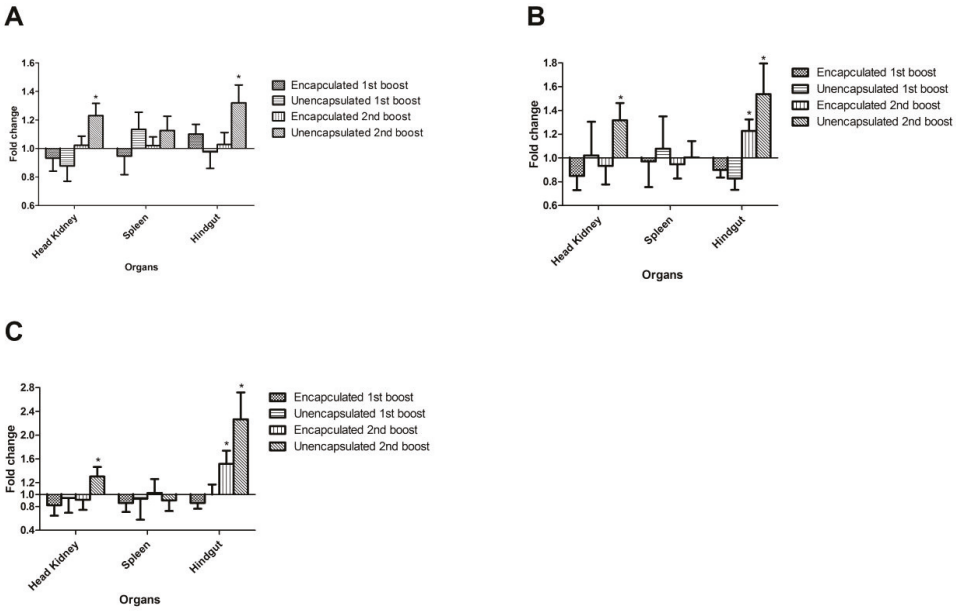


Figure 9. Mean relative expression of A) FOXP3; B) TGF-β and C) IL-10 genes in fish orally boosted with different antigen preparations compared to un-boosted controls. n=30; *statistically significant $p< 0.05$.

10.8 The correlation between antigen kinetics and immune responses

To our knowledge, the correlation between antigen kinetics and immune response following oral administration is in general poorly understood. In this thesis, IPNV antigens were detected in situ in leukocyte-like cells in the hindgut, head kidney and spleen (paper II), and immune responses were induced in these organs (paper III). The results suggest that the oral vaccine can induce not only local but also systemic humoral immune responses since antigens translocate across the gut to systemic distribution/circulation and then transmitted via the circulatory system to the immune organs. The findings are indicative of the immune responses lasting up to the second oral boost (paper III), possibly aligned with the IPNV antigens being associated with MMCs, providing long-term antigen retention (paper II).

There are few studies (to my knowledge) showing uptake of IPNV antigens from the gut (given orally) and the antigen kinetics revealed in paper II thus provide more insight into kinetics of oral vaccination of fish. Further studies of the relationship between antigen kinetics and immune responses would help us improve the efficacy of oral vaccines. For example, the induction of immune tolerance (paper III) was likely influenced by the dose and formulation,.

11 Conclusion

Antigen production

- The high cell numbers and prolonged survival of AGK cells are probably an important factor for the superior virus yields in AGK cells.
- The higher split ratio/shorter split interval makes AGK superior over other available cell lines.

Antigen kinetics

- Inactivation of IPNV by formalin did not influence the IPNV antigen kinetics following oral intubation.
- The hindgut not the foregut is the segment of intestine where IPNV antigen uptake in Atlantic salmon.
- The antigens could indeed be taken and delivered to the immune organs, including the macrophage-like cells and MMCs, which play important roles in immune system of fish.

Immune response

- Parenteral prime with oil-adjuvant vaccine followed by oral boost with ENCAP results in an augmentation of both systemic and mucosal immune responses.
- Oral boost with ENCAP results in transient augmentation of mucosal immune responses.
- Oral boost with UNENCAP results in the induction of immunological tolerance.

12 Perspectives

Antigen production

- Since the characteristics of AGK cell line enhance the production of virus, it may also be useful for producing other virus such as fish alphavirus. Therefore, it will be beneficial to understand if AGK cells can be used to produce other virus with high yield for vaccine.

Antigen Kinetics

- Further understanding of antigen kinetics will be a fascinating subject to study people to develop vaccine or to optimize the method to assess the feasibility of vaccine development.

Efficacy of oral vaccine

- Performing a challenge test will be the next and ultimate test aimed to show the effect of a boost regime. Previous reports indicate that the level of antibody can be used to predict the protection of vaccine [35].
- It will be necessary to detect IgT levels in intestine and other mucosal surface post oral vaccination.
- A very interesting and yet poorly understood mechanism is the contrasting difference in oral immune tolerance in non-formulated and alginate formulated vaccine formulations. To what this applies to other antigens and also will impact protection against challenge remains to be studied and elucidated.

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Delayed protein shut down and cytopathic changes lead to high yields of infectious pancreatic necrosis virus cultured in Asian Grouper cells

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Inactivated whole virus vaccines represent the majority of commercial preparations used to prevent infectious pancreatic necrosis (IPN) in salmonids today. The production of these vaccines requires high virus concentrations that are resource-demanding. In this study, we describe the cultivation of high yields of IPN virus in Asian Grouper strain K (AGK) cells. The mechanism by which this is achieved was investigated by comparison with commonly used salmonid cell lines (RTG-2 and CHSE-214 cells). The cells were counted before and sequentially after infection. Thereafter, protein shut down, virus yields and apoptosis were assessed. The effects of poly(I:C) pre-treatment and Mx expression on IPNV concentrations were examined and the results show that high virus yields were associated with high cell numbers per unit volume, delayed cell death and apoptosis in AGK cells while the opposite was observed in RTG-2 cells. Poly(I:C) treatment and Mx expression resulted in a dose-dependent inhibition of virus multiplication. The production capacity of AGK and CHSE-214 cells were compared and higher split ratio and shorter split interval of AGK cells documents dramatic differences in virus antigen production capacity. Collectively, the results suggest that high cell numbers and prolonged survival of AGK cells are responsible for the superior virus yields over RTG-2 and higher split ratio/shorter split interval makes AGK superior over CHSE cells.

1. Introduction

Infectious pancreatic necrosis (IPN) is one of the most important diseases of farmed salmonids the world over. Originally known to affect fry at the point of start-feeding and in smolts shortly after sea water transfer, the disease now afflicts fish at all stages of production (Roberts and Pearson, 2005). IPN is caused by an un-enveloped double stranded RNA virus, the IPN virus (IPNV) that is a prototype of the genus Aquabirnavirus in the family *Birnaviridae* (Cohen et al., 1973; Duncan and Dobos, 1986). The virus genome consists of two segments, A and B, with the former encoding structural proteins VP2 and VP3, and non-structural proteins VP4 and VP5 (Dobos, 1976; Havarstein et al., 1990). Segment B encodes VP1, the RNA dependent RNA polymerase (Duncan et al., 1991).

Disease prevention and control of IPN include vaccination of parr during the fresh water stage with the purpose to protect them against disease during the first 2 months post sea transfer. Several vaccines are available, most of which are based on inactivated whole virus but also on subunit preparations (Sommerset et al., 2005) although their performance is equivocal. At experimental level, inactivated whole virus vaccines have shown better protection than recombinant subunit vaccines, the former holding promise as effective vaccines for the future (Munang'andu et al., 2012, 2013a). The efficacy of the vaccines relies on inclusion of high antigen content.

Several cell lines have been tested for their suitability in the propagation of IPNV (Lannan et al., 1984) and are indeed in use in several laboratories for example rainbow trout gonad 2 (RTG-2) and Chinook salmon embryo 214 (CHSE-214) cells. The yields of virus that these cell lines give differ between cell lines and even between virus isolates. In general, the Sp serotype yields lower quantities (10^6–10^7 pfu/ml) in RTG-2 cells compared to CHSE-214 (10^8 pfu/ml) or even up to 10^{10} $TCID_{50}$/ml (Skjesol et al., 2009). The difference in yields between RTG-2 and CHSE-214 cells has been attributed to the former's ability to elicit an interferon (IFN) response believed to be absent in the latter (Macdonald and Kennedy, 1979; Jensen et al., 2002). Other factors including apoptosis and necrosis as well as the rates at which they occur may also contribute to the virus yield although this is not well documented. As stated above, vaccine production, challenge models, immunological and biological assays demand the cultivation of virus in large quantities. We report the use of Asian Grouper strain K (AGK) cells that support the cultivation of high titres of IPNV (10^9–10^{10} $TCID_{50}$/ml) (Munang'andu et al., 2012). These cells are derived from the skin of a crossbreed between Orange-spotted grouper (*Epinephelus coioides*) and Malabar grouper (*Epinephelus*

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malabaricus). The cell line is not a mono-clone but is composed of different cell types. AGK cells grow at a higher temperature, can be split at high ratios and have a much shorter turn-around time making them a very valuable resource for easy production of IPNV in large quantities within a short period of time.

The purpose of the present study was to investigate and document the factors that permit the production of high titres and high volumes of IPNV using AGK cells. A good understanding of these factors will contribute to more efficient production of antigens that are required in the development of protective vaccines against IPNV.

2. Materials and methods

2.1. Cells

Rainbow trout gonad 2 cells (RTG-2; ATCC CCL-55) were maintained at 20 °C with L-15 medium (Invitrogen) supplemented with 10% foetal bovine serum (FBS), 10% L-glutamine and 1 μl/ml of gentamicin. Asian Grouper strain K (AGK) cells (Munang'andu et al., 2012) were maintained at 28 °C with L-15 media of the same composition but supplemented with 7.5% FBS while Chinook salmon embryo cells (CHSE-214; ATCC CRL-1681) were maintained at 20 °C also with the same medium but containing 10% FBS.

2.2. Experimental design

RTG-2 and AGK cells were used to grow IPNV as a basis for understanding the mechanisms underlying differences in yields. CHSE-214 cells were used to quantify virus yields from both cell lines.

2.3. Assessment of IPNV yields in AGK and RTG-2 cells

A recombinant IPNV strain (rNVI-15PT) with amino acids Proline (P), Threonine (T) and Alanine (A) in positions 217, 221 and 247 in the VP2 protein, respectively (Munang'andu et al., 2013b), was used to infect confluent cells. The cells were infected in triplicates by first adsorbing the virus for 2 h on a rocking board, then washing with PBS before replacing fresh maintenance media and incubating them at 15 °C. Virus samples from both intact cells and culture supernatants were harvested in parallel, on daily basis until full CPE. The virus yield in these samples was assessed by titration in CHSE-214 cells.

To assess the effect of virus to cell infection ratio on virus yields, the cells were infected with either MOI = 0.1 or MOI = 10. Virus yields were assessed as described above. In addition, quantitative RT-PCR was also used to assess virus yields of AGK cells from day 1 to 5.

2.4. Metabolic labelling of newly synthesized proteins

AGK and RTG-2 cells infected with IPNV (MOI = 0.1 and 10) were incubated for the required duration (up to 5 days maximum). To harvest the cells, they were first washed with PBS and then incubated for 1 h in Dulbecco's modified Eagle's medium without Methionine and supplemented with 0.1% FBS and 20 Ci [^{35}S]Methionine/ml. After incubation, the cells were washed three times with PBS and then lysed by using CelLytic M reagent (Sigma). The protein was separated by SDS-PAGE and blotted onto a poly(vinylidene) fluoride (PVDF) membrane. The membrane was then kept in a Phosphor cassette over-night prior to scanning using a Typhoon imager (GE Healthcare).

2.5. Transfection of poly(I:C) in AGK and RTG-2 cells followed by IPNV infection

To examine antiviral effects, AGK and RTG-2 cells were grown in 6-well culture plates until near confluence. Thereafter they were transfected with 3 μg of poly(inosinic:polycytidylic) acid (poly(I:C)) and 9 μl of FuGENE® HD Transfection Reagent (Roche) per well according to the manufacturer's instructions. 24 h later, the cells were infected with IPNV at MOI of 0.1. At 48, 72 and 96 h following infection, the viral proteins were assessed by Western blot.

2.6. Assessment of Mx expression following IPNV infection in AGK cells

AGK cells were infected with IPNV (MOI of 0.1 and 10). Sampling was done daily for 6 days. Total RNA was isolated by using the RNeasy Plus mini kit (Qiagen), and the concentration of RNA was determined by spectrophotometry (Nanodrop ND1000). For each sample, 500 ng of total RNA was subjected to cDNA synthesis using Transcriptor first-strand cDNA kit (Roche) in a total volume of 20 μl as described above. Mx expression was determined by quantitative real time PCR (qPCR) and this was performed by using Light-Cycler 480 SYBR green I Master mix on a LightCycler 480 thermocycler (Roche). 2 μl of cDNA was used as a template in a final volume of 20 μl. The mixtures were first incubated at 95 °C for 10 min, followed by 40 amplification cycles (10 s at 95 °C, 20 s at 60 °C, and 8 s at 72 °C).

The sequences of primers (Mx-574 Fwd and Mx-730 Rev) used to assess the expression of Mx are given in Table 1. The specificity of the PCR products from each primer pair was confirmed by melting-curve analysis and subsequent agarose gel electrophoresis. The $2^{-\Delta\Delta CT}$ method was used to calculate the amount of gene products as described previously (Nolan et al., 2006; Schmittgen and Livak, 2008). $2^{-\Delta\Delta CT}$ is the relative mRNA expression representing the fold induction over the control group. All quantifications were normalized to Cathepsin D. This gene has been demonstrated not to be induced in IPNV-persistently infected CHSE-214 cells (Marjara et al., 2010). In the present study, Cathepsin D expression was tested in AGK cells and found not to be induced by infection with IPNV.

2.7. Over-expression of Mx in AGK cells

The full Mx gene sequence (GenBank Accession No. KF148054) was amplified by PCR using the primers pcDNA3.1c-Mx-F and pcDNA3.1c-Mx-R (Table 1) containing BamHI/EcoRI restriction sites. A truncated form of the gene was used as a negative control and was produced using primers pcDNA3.1c-MxN-F and pcDNA3.1c-MxN-R. PCR was done as described above. 3 μg of PCR products and pcDNA3.1c vector were digested by using restriction enzyme BamHI and EcoRI (Promega) for 3 h and were directionally ligated using a ratio of 1:3. The plasmids were sequenced to confirm orientation and position of the gene as well as that of the His-tag.

Transfection of AGK cells was done in 6-well plates using 3 μg of plasmids containing the Mx gene and 9 μl of FuGENE® HD Transfection Reagent (Roche) per well according to the manufacturer's instructions. After 48 h, the cells were infected with IPNV (MOI of 0.1). 24 h post infection, the Mx and viral protein expression were detected by IFAT.

2.8. Simultaneous assessment of Mx over-expression and IPNV infection in AGK cells

AGK cells over-expressing Mx or not (controls), grown in 6 well plates were infected with IPNV as described above. At 72 h post-infection, the culture medium was removed from the cells, washed

Table 1
List of primers used in this study.

Name	D*	Sequence (5′–3′)	T_A (°C)
MxC-f	F	MAGGGACARCCAGARRACATTGGA	59.7
MxC-r	R	AAYYAAMYTSCTGCTGTAKGTSCTGT	59.4
Mx3Race		TCC AAC CTA GGC AGC GAT TTC TCA	60.1
Mx5Race		TTG GCC TTG GCA GAT TTG AGC AGA	61.3
Mxfull-f	F	ATGACAAACTCAACAGAAGATCAGCTG	57.3
Mxfull-r	R	TTAGGATCCAAACTCCAGCAGGTAGCT	61.0
pcDNA3.1c-Mxf	F	GAATTCTGCCGCCATGACAAACTCAACAGAAGATCAGCTG	65.8
pcDNA3.1c-Mxr	R	GGATCCAAACTCCAGCAGGTAGCTGCGTGC	67.1
pcDNA3.1c-MxNf	F	GAATTCTGCCGCCATGACAAACTCAACAGAAGATCAGCTG	65.8
pcDNA3.1c-MxNr	R	GGATCCAAACTCCAGCAGGTAGCTGCGTGC	67.1
Mx-574	F	TTGAAGATGGCACAGGAGGTGGAT	60.3
Mx-730	R	TGCACCTGACAATCATGTAGCCCT	60.5
Cathepsin D-f	F	CAGGCTGGTAAGACCATCTGC	57.8
Cathepsin D-r	R	TGTTGTCACGGTCGAACACAG	57.6

T_A, annealing temperature; D*, direction of primer.

with PBS and then fixed for 20 min in 4% paraformaldehyde solution (Sigma, USA) at room temperature. After washing twice for 5 min with PBS, incubation was performed for another 5 min on ice with 200 μl/well of 0.1% Triton X-100 (Sigma). After washing as described above, the cells were then blocked with 5% BSA for 20 min at room temperature. Following removal of the blocking solution, primary antibodies (mouse anti-polyhistag (Sigma)) or rabbit anti-IPNV (Evensen and Rimstad, 1990) diluted 1:1000 in 2.5% BSA were added and incubated for 30 min at room temperature. The wells were then washed before adding secondary antibodies (IgG goat anti-mouse Alexa Flour 488 or goat anti-rabbit Alexa Flour 594 (Invitrogen)) diluted 1:1000 in 2.5% BSA. The samples were incubated for 30 min at room temperature before the final washing prior to examination by fluorescence microscopy.

2.9. Assessment of apoptosis in AGK and RTG-2 cells following infection with IPNV

Cells were gently trypsinized and washed once with L15 medium containing 7.5% FBS prior to collection in tubes by centrifugation at 300 × *g* for 10 min. They were then re-suspended in 1× binding buffer of Annexin-V-FLUOS Staining Kit (Roche). The supernatant was transferred to a 5 ml polystyrene round-bottomed tube (BD Biosciences). Thereafter, 2 μl of Annexin V-FITC was added and the cells incubated at room temperature for 30 min in the dark. Thereafter the volume was adjusted to 300 μl. Propidium iodide (PI) was added at 8 μl/ml just before analysis. The BD FACS Aria cell sorter (BD, San Jose, CA) was used for the analysis with Annexin V-FITC binding with FITC signal detector and PI staining by the phycoerythrin emission signal detector. Data analysis was performed using BD FACS DiVa Software, version 5.0.2 (BD, San Jose, CA). Cell aggregates were identified and excluded by using the width pulse of FSC-A versus area width of SSC-A. The percentage of early and late apoptotic cells were calculated for each sample.

2.10. Statistical analysis

Data were analyzed for statistical significance using a two-tailed Student's *t* test assuming populations of unequal variance. The threshold for significance was $p < 0.05$.

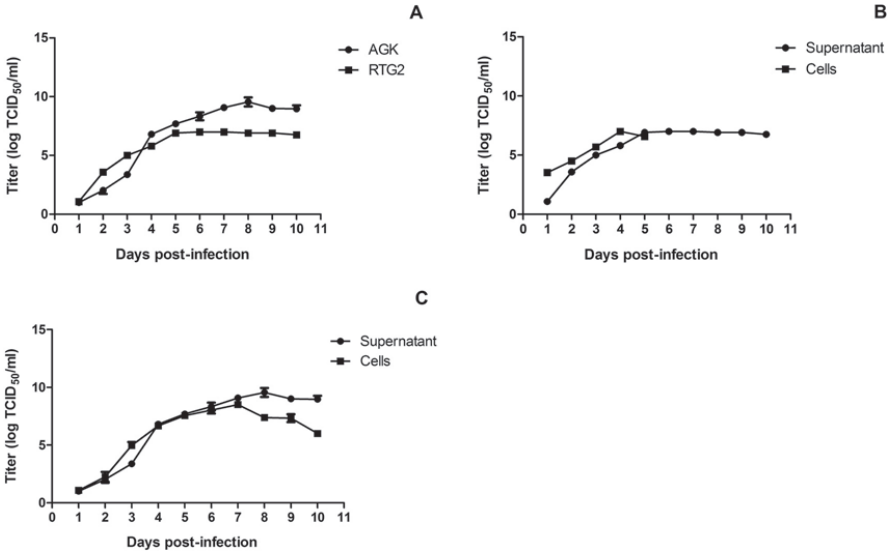

Fig. 1. Infectious pancreatic necrosis virus (IPNV) yields in AGK and RTG-2 cells. Virus concentration in supernatants of AGK and RTG-2 cells infected at MOI = 0.1 (A). Time-course concentrations of virus recovered from cells versus supernatants of RTG-2 and AGK cells are shown by (B) and (C), respectively.

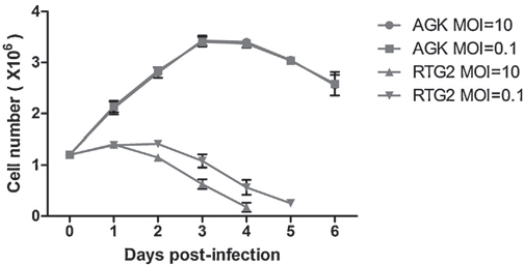

Fig. 2. Numbers of AGK and RTG-2 cells over time post infection with infectious pancreatic necrosis virus at MOI of either 0.1 or 10.

3. Results

3.1. Infection of AGK cells with IPNV results in delayed CPE and higher virus yields compared to RTG-2 cells

Full CPE was observed at 7 days post infection (d.p.i.) in RTG-2 cells compared to 10 d.p.i. in AGK cells. The time course assessment of virus yields harvested from cells or supernatants and then titrated in CHSE-214 cells showed higher concentrations of IPNV propagated in AGK compared to RTG-2 cells (Fig. 1A). As expected, the virus concentration was initially higher in intact cells compared to the supernatants but this became reversed towards full CPE (Fig. 1B and C). At full CPE, the virus yield in AGK cells was significantly higher ($p < 0.01$) than in RTG-2 cells (Fig. 1A–C). In order to investigate the basis for this difference, the numbers of cells in infected AGK and RTG-2 cells were counted from day 0 to 6 post infection in parallel wells by using a Countess® Automated cell counter (Invitrogen). In both cells types, there were about 1.2×10^6 cells/ml at the time of inoculation (Fig. 2). The highest average number of infected AGK cells attained was about 3.5×10^6/ml at 3 d.p.i. irrespective of the MOI used. In contrast, the highest average number of RTG-2 cells was only 1.3×10^6/ml at 1 d.p.i.

3.2. Protein shut down in AGK cells is delayed compared to RTG-2 cells following infection

To find out why it takes longer to achieve full CPE in AGK compared to RTG-2 cells, protein synthesis following infection was examined. [^{35}S]Methionine-labelled host protein synthesis revealed reductions (shutting down of protein synthesis) in both cell types, but much earlier in RTG-2 cells compared to AGK cells. In the former, protein shut down started as early as 1 d.p.i. in cells infected with virus at MOI = 10 (Fig. 3). In these cells, virus synthesis visualized as strong protein bands were observed. At 2 d.p.i., the virus bands were stronger in RTG-2 cells while host proteins were almost completely shut down. In contrast, no reduction in protein synthesis was observed in AGK cells prior to 5 d.p.i., even in cells inoculated with MOI = 10 (Fig. 3). However, strong viral protein bands were visible from 3 d.p.i. onwards irrespective of the virus MOI used for infection.

3.3. Poly(I:C) transfection prior to infection delays virus growth in AGK

To address questions of delayed production of viral proteins and prolonged survival of AGK cells, innate antiviral responses were examined. Since recombinant interferon-α (IFN-α) of grouper was not available to us, poly(I:C), a known inducer of IFN-α was used to transfect cells 24 h prior to infection. The response was assessed indirectly by monitoring viral bands sequentially by Western blot. The same was done in RTG-2 cells for comparison. Fig. 4A shows that no difference was observed in the expression of virus bands between poly(I:C) transfected and untreated RTG-2 cells. In contrast, AGK cells transfected with poly(I:C) exhibited delayed virus protein expression, with bands first appearing at 4 d.p.i. compared to 2 d.p.i. in non-poly(I:C)-transfected controls (Fig. 4B). However, from 4 d.p.i. onwards, no differences between groups was observed, suggesting that poly(I:C) delays but does not prevent replication of IPNV in AGK cells.

3.4. Kinetics of Mx expression in AGK cells infected with IPNV

To further examine antiviral responses, the effect of Mx downstream of the IFN response was examined. Mx was constitutively expressed in uninfected control group albeit at low levels (not shown). In infected cells, the expression exhibited a biphasic induction pattern with only cells inoculated with virus at MOI = 10 showing significant induction ($p < 0.01$) at 1 d.p.i. (Fig. 5). Notably, significant induction was only observed in cells infected with MOI = 10 while significant down-regulation was in cells infected with virus at MOI = 0.1 (Fig. 5).

To understand the relationship between Mx expression and IPNV replication better, cells were infected at MOI = 0.1 and 10, and the virus was quantified over time post infection. The virus

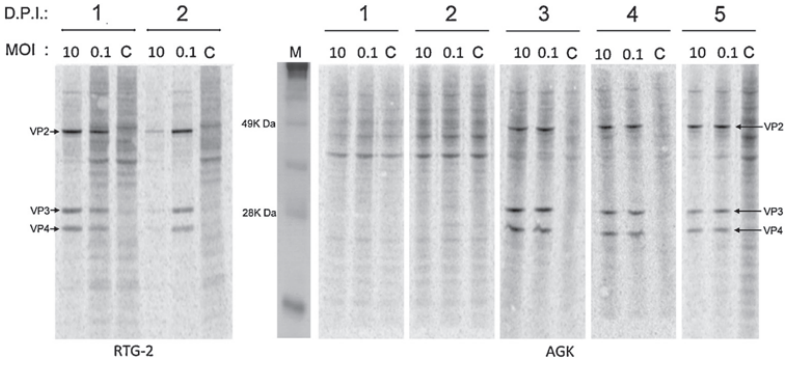

Fig. 3. Protein shutdown in RTG-2 and AGK cells following infection with infectious pancreatic necrosis virus. Key: VP2, VP3, VP4 = virus proteins; M = marker; d.p.i. = days post infection; MOI = multiplicity of infection, C = negative control. Newly synthesized proteins were labelled with [^{35}S]Methionine, separated by SDS-PAGE, blotted on PVDF membrane and imaged using a Typhoon imager.

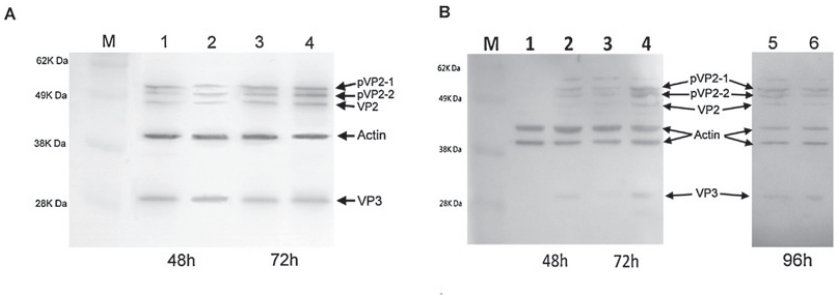

Fig. 4. Western blot of infectious pancreatic necrosis virus capsid proteins in AGK cells transfected with poly(I:C) prior to infection. Key: M: marker; 1, 3, 5 = poly(I:C) treated cells and 2, 4, 6 = untreated controls at indicated time points; pVP2-1, pVP2-2 = precursor proteins of the virus VP2; VP2 and VP3 = virus capsid proteins.

yield recovered from intact cells was assessed sequentially by titration in CHSE-214 cells. For RTG-2 cells, a difference (higher yields with MOI = 10 compared to MOI = 0.1) was observed during the first 3 days only (Fig. 6A). Thereafter, no difference in yields was observed. In contrast, there was no difference in yields between groups (MOI = 0.1 or 10) at early times (days 1–5) for AGK cells (Fig. 6B). At later times (days 5–10) however, higher virus yields in cells infected with MOI = 0.1 compared to 10 (which was opposite to the Mx expression (Fig. 5)) was observed. Thus in order to contrast between the virus quantities better, qRT-PCR was used on samples collected from days 1 to 5. Fig. 6C shows that at 1 d.p.i., the virus concentration in intact cells (infected at MOI = 10) was significantly higher ($p < 0.01$) than that of MOI = 0.1, coinciding with the expression pattern of Mx (Fig. 5). However, by day 3, the difference had evened out while as from day 4 onwards, the virus concentration was higher in cells infected with MOI = 0.1 than MOI = 10 (Fig. 6C).

3.5. AGK cells over-expressing Mx are protected against IPNV infection

In order to specifically test the antiviral effects of AGK Mx, the gene was over-expressed in AGK cells prior to infection with IPNV and the effect was assessed by IFAT. The Mx protein was fused with a His tag (detected using anti-polyhistag antibodies) while IPNV was detected by using anti-IPNV antibodies. The transfection efficiency was on average relatively low, however the cells that received the construct were in general protected against IPNV infection (Fig. 7A) although a few cells co-expressing both Mx and IPNV were also observed. In contrast, cells over-expressing the truncated form of Mx (negative control) were not protected (Fig. 7B).

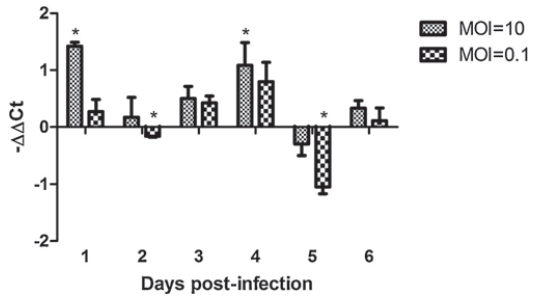

Fig. 5. Relative mRNA levels of Mx in AGK cells following infection with infectious pancreatic necrosis virus at either MOI of 0.1 or 10 as measured by real-time quantitative RT-PCR. *Significantly different (at least $p < 0.05$).

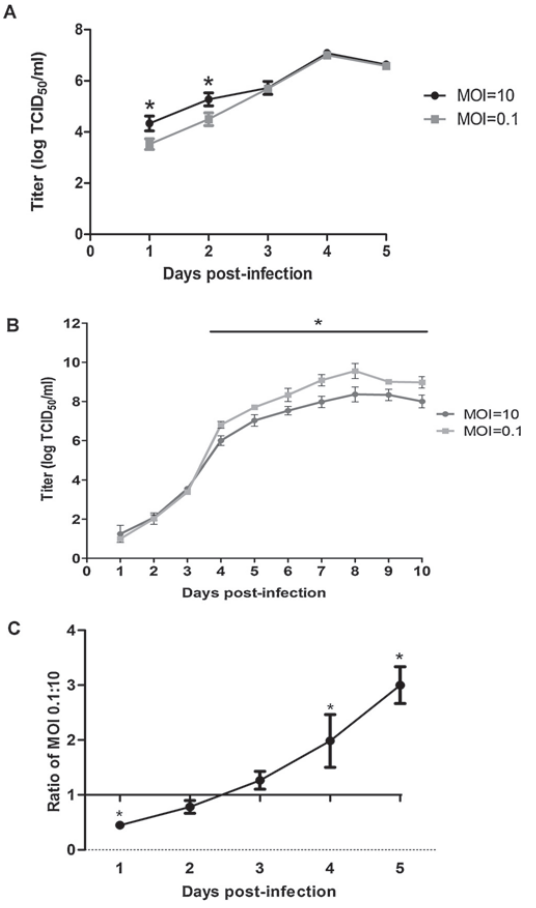

Fig. 6. Infectious pancreatic necrosis virus yields harvested from AGK and RTG-2 cells infected either at MOI 0.1 and 10. Average concentrations of the virus in (A) RTG-2 cells and (B) AGK cells assessed by titration in CHSE-214 cells. (C) Average concentrations of the virus in AGK cells assessed by real time RT-PCR. *Significantly different (at least $p < 0.05$).

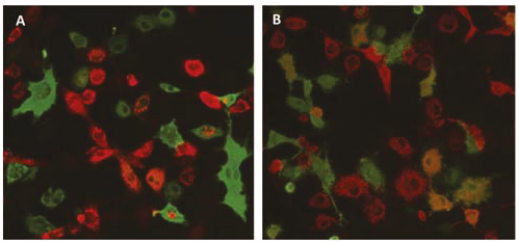

Fig. 7. Indirect fluorescence antibody test of overexpressed Mx (A) in AGK cells following infection with infectious pancreatic necrosis virus at 72 h post infection. (B) Negative control transfected with the truncated form of Mx, note the degree of double staining. Key: green colour = Mx; red stain = IPNV; yellow/orange = double staining of IPNV and Mx(A) or its trunchated form(B).

3.6. IPNV-infected AGK cells are less apoptotic than RTG-2 cells

Other factors likely to contribute to cell survival or the lack of it following virus infection include apoptosis and necrosis. To determine which one was applicable in AGK and RTG-2 cells infected with IPNV, the cells were subjected to flow cytometry. At 24 h following infection, a higher percentage of infected RTG-2 cells (9.6%) underwent apoptosis compared to AGK cells (0.6%) (Fig. 8).

3.7. AGK yields much higher volumes of virus supernatant than CHSE over the same time period

While the studies above provide some explanation as to why AGK cells yield a higher virus titre than RTG-2 cells, it has been shown that titres equivalent to those offered by AGK cells can be obtained in CHSE-214 cells (Skjesol et al., 2009, own observation). For this reason we extended this study and compared the growth capacity of AGK and CHSE-214 cells in 162 cm^2 flasks. Both cell lines were seeded onto 162 cm^2 with 3.7×10^6 cells as a start and incubated at their optimum temperatures (28 °C for AGK and 20 °C for CHSE-214 cells). AGK cells became confluent and were ready to be split after 3 days while CHSE-214 only became confluent after 7 days of incubation. AGK cells have a split ratio (SR) of 1:4 and CHSE are split at 1:3. On this basis we calculated how many flasks can be obtained after 3 weeks of culture, using the following formula; number of flasks = $SR^{(no.\ splits)}$. The number of flasks of AGK after 3 weeks (6 splits) will be 4096, for CHSE 27 flasks (3 splits). This will be equivalent to 163 840 ml of supernatant for AGK and 1080 ml for CHSE (40 ml per bottle in a 162 cm^2 flask). At a virus yield of 1.0×10^9 $TCID_{50}$/ml of supernatant (for both) and a vaccine dose of 5×10^8 $TCID_{50}$/fish, AGK cell production will support the vaccination of 327 680 fish while only 2160 fish can be vaccinated from CHSE-grown virus.

4. Discussion

It was shown in this study that AGK cells produce higher yields of IPNV compared to RTG-2 cells. IPNV titres in excess of 10^9 $TCID_{50}$/ml were obtained from the former (Fig. 1), translating to about 100 times more than the yield of RTG-2 cells and comparable to previous records of CHSE-214 and CHH-1 (Lannan et al., 1984; Skjesol et al., 2009). When comparing production capacity to CHSE cells, the higher split ratio and shorter split interval of AGK cells will give a very marked difference in production capacity. Infected AGK cells had more than twice the number of RTG-2 cells per unit volume at confluence (Fig. 2), a difference probably explainable by their smaller size in comparison with the latter. This means that AGK cells can reproduce more than RTG-2 cells. It is expected that the large number of AGK cells provide more cells for the virus to multiply leading to higher yields.

AGK cells grow more rapidly compared to salmonid cell lines probably because of their relatively higher physiological and in vitro temperature tolerance (28 °C). Temperature has previously been demonstrated to induce increase growth rate in mammalian cells through its effect on the G1 growth phase (Watanabe and Okada, 1967). AGK cells are split at 1:4, not very different from CHSE-214 cells (1:3), but they grow confluent in 3 days as opposed to 7 days (CHSE-214 cells). The effect of higher split ratio and the shorter split interval demonstrate the advantage these cells have over CHSE-214 and other similar salmonid cell lines. From a vaccine production point of view, the effect of shorter splitting intervals results in a very high number of flasks available for inoculation and the resulting vaccine volume will differ dramatically.

While the difference in cell numbers between AGK and RTG-2 cells can only account for part of the difference in yields, the rest can be attributed to individual cells' virus carrying capacities, likely facilitated by delayed protein shutdown and delayed/lack of apoptosis in AGK cells. Protein shutdown is a well-known mechanism employed by cells to protect themselves against virus infections (McClintock et al., 1986; Guzo et al., 1992; Du and Thiem, 1997). It is also used by viruses to subvert host antiviral responses (Mazzacano et al., 1999; Mir and Panganiban, 2008). IPNV has previously been shown to interfere with host protein synthesis (Skjesol et al., 2009) although the mechanism has not been clearly elucidated. In this study, the delay in protein shutdown of AGK cells (Fig. 3) was associated with high host cell numbers and virus yields. This is in contrast to the findings of RTG-2 cells where cell multiplication ceased at 1 d.p.i. (MOI = 10) or 2 d.p.i. (MOI = 0.1) (Fig. 2), coinciding with host cell protein shutdown. Furthermore, apoptosis was detected in RTG-2 cells by 1 d.p.i. unlike in AGK cells where it was absent at this time point. Collectively, these findings suggest that delayed protein shutdown and CPE in AGK cells allows for longer replication windows that in turn permit increased virus yields, opposite to what is seen in RTG-2 cells.

The similarities in the trends of protein shutdown of AGK cells infected with different MOI in this study (Fig. 3) were intriguing given that the final virus concentrations were different (Fig. 6). This is opposite to what was seen in RTG-2 cells where shutdown was enhanced by high MOI and the virus yield ultimately ending up with the same concentration for both high and low MOI. In AGK cells, the low expression of Mx, while constitutive, did not inhibit IPNV multiplication. Infecting cells with high MOI triggered the induction of Mx leading to reduced final yields. In contrast, inoculation of cells with low MOI induced only marginal non-significant changes in the Mx expression, ultimately with higher virus concentrations. Taken together, these findings support previous reports that the effect of Mx on IPNV is dose-dependent, the more the expression, the more its antiviral effects (Lester et al., 2012). Low MOI allows the virus to multiply without inducing Mx and results in high virus yields.

The production and responsiveness of interferon (IFN) by some cell types, for example RTG-2 has previously been shown to result in low virus concentrations while the absence has been linked to high titres, e.g. in CHSE-214 cells (Dobos and Roberts, 1983). Cellular IFN is produced by infected cells and acts on neighbouring cells by inducing the production and release of many interferon stimulated genes (ISGs) including Mx to create an antiviral state (Galligan et al., 2006). Poly(I:C), a potent inducer of interferon was used in the present study to transfect both AGK and RTG-2 cells (Fig. 4) (Skjesol et al., 2009). While no difference between groups was observed in RTG-2 cells, poly(I:C) transfection initially inhibited virus replication in AGK cells. These results fit well with those of Mx expression following IPNV infection with MOI = 10 (Fig. 5) and strengthen the argument that interferon delays but does not inhibit IPNV multiplication as shown previously (Skjesol et al., 2009). Unfortunately, the

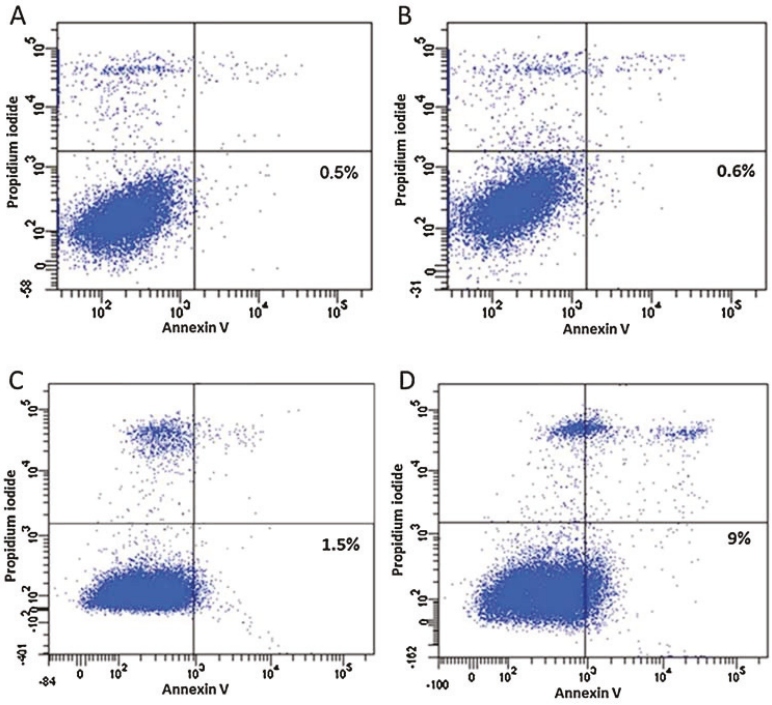

Fig. 8. Flow cytometry analysis of AGK and RTG-2 cells at 24 h post infection with infectious pancreatic necrosis virus. (A) Uninfected AGK control cells; (B) IPNV-infected AGK cells; (C) uninfected RTG-2 control cells and (D) IPNV-infected RTG-2 cells. Apoptotic cells are strong Annexin V positive while necrotic cells have increased propidium iodide stain.

genetic sequence of AGK IFN is not known at present, nor are there tools that can be used to study the interferon response directly although the anticipation is that the IFN response in AGK cells is conserved.

IPNV kills infected cells by lysis, referred to as post-apoptotic necrosis in fish cell lines (Hong et al., 1998, 1999; Santi et al., 2005). It is not very clear however whether apoptosis is part of the virus pathogenesis mechanism. At least one report suggests that it is part of the host cell antiviral mechanisms (Imajoh et al., 2005). In the present study, more apoptosis was observed in RTG-2 compared to AGK cells at 24 h p.i. These results are consistent with those of protein shutdown and support the observation that infected AGK cells remain viable longer than AGK cells following infection. However, additional studies are required to document the contribution of apoptosis or the lack of it to virus yield.

IPNV has been propagated previously in AGK cells in our laboratory for both inactivated whole virus vaccines and challenge models, achieving strong protection against homologous challenge in experimental models where mortalities of 84.6% in naïve fish were obtained (Munang'andu et al., 2012, 2013a,c). This clearly shows the potential of the AGK cell line used for production of IPN vaccine antigen.

Acknowledgements

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II

IPNV antigen uptake and distribution in Atlantic salmon following oral administration

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Abstract

One of the difficulties facing the success of oral vaccination in fish is the hostile stomach environment that antigens must cross. Further, uptake of antigen from the gut to systemic distribution is required for induction of systemic immunity, the dynamics of which are not well understood. In the present study, groups of Atlantic salmon parr were intubated with live or inactivated IPNV either orally or anally. At 1, 24 and 72hrs post infection (p.i.) the fish were sacrificed and serum, head kidney, spleen, liver, anterior and posterior intestinal tissues were sampled. The serum was used for assessing IPNV by ELISA while tissues were used for antigen distribution by immunohistochemistry. Both live and inactivated IPNV antigens were observed in enterocytes of the intestines and in immune cells of the head kidneys and spleens of all groups. In the liver, no antigens were observed in any of the groups. Significantly higher serum antigen OD

values ($p<0.04$) were observed in orally compared to anally intubated fish. By contrast, no difference ($p=0.05$) was observed in tissue antigens between these groups by immunohistochemistry. No significant difference ($p=0.05$) was observed in serum antigens between groups intubated with live and inactivated IPNV while in tissues, significantly more antigens ($p<0.03$) were observe in the latter compared to the former. These findings demonstrate that both live and inactivated IPNV is taken up by enterocytes in the intestines of Atlantic salmon likely by receptor mediated mechanisms akin to those of HRP or ferritin. Higher IPNV uptake by the oral compared to anal route suggests that both the anterior and posterior intestines are important for the uptake of the virus and that IPNV is resistant to gastric degradation of the Atlantic salmon stomach.

Key words

Infectious pancreatic necrosis virus, Atlantic salmon, oral, anal, uptake

Introduction

Oral vaccines are the most desirable preparations for use in the aquaculture industry for several reasons: they are stress-free, can be mass-applied to fish of any size and are not labour intensive [1-3]. Despite these advantages, only a few commercial preparations are available on the market at the moment including those against infectious pancreatic necrosis virus (IPNV), Spring viremia carp virus (SVCV), infectious salmon anaemia virus (ISAV) and *Piscirickettsia salmonis* [4;5]. This status quo highlights the market potential for oral vaccines in the aquaculture industry but also reflects the challenges faced in their development.

43 One of the problems associated with oral vaccination of fish is the poor induction of local and
44 systemic immunity by the vaccines. Indeed oral vaccines come third after injection and
45 immersion preparations in terms of efficacy [6]. Previous studies suggest that this is a result of a)
46 antigen destruction from exposure to gastric acids and digestive enzymes in the gut of some
47 species of fish, b) poor uptake of antigens over the intestinal epithelium and c) induction of
48 tolerance following oral administration [7;8]. Therefore to resolve some of these obstacles,
49 several encapsulation formulations with the ability to protect the antigens through the hostile
50 environment of the stomach have been developed such as alginate beads or microspheres [6;9].
51 Nevertheless, even with these formulations, variable results in the vaccination of fish have been
52 reported with different antigen preparations [9;10]. It is also noteworthy that the assessment in
53 these studies were done mainly by examining mortalities or survival of fish following challenge
54 (summarized in [6]) whilst antigen uptake remains poorly understood. In the present study, we
55 examined the uptake and distribution of IPNV at early time in selected organs following oral and
56 anal intubation. This has previously not been well documented. Novoa and coworkers attempted
57 to show the uptake and sequential distribution of IPNV in turbot following intraperitoneal
58 injection and immersion infection and drew conclusions at tissue level, but failed to document
59 which cells take up the virus at the portals of entry [11].

60 Infectious pancreatic necrosis is a disease caused by IPNV and affects salmonids especially fry at
61 start feeding, parr during fresh water and post-smolts a few weeks after seawater transfer. IPNV
62 uptake in fish has in general not been studied in detail since the 1990's. Available literature
63 shows that the second gut segment is important for uptake of proteins following both oral and
64 anal administration [10;12;13]. For IPNV however, Sundh and colleagues found that both
65 proximal and distal intestines were routes of uptake in Atlantic salmon [14]. In carp, HRP (solid

66 phase) is taken up by the receptor mediated route and is sorted into the endolysosomal
67 compartment and intercellular spaces [7]. Ferritin and LPS (fluid phase) on the other hand are
68 taken up through the large supranuclear vacuoles and cannot be observed in intracellular spaces
69 [10;12;13]. How IPNV is taken up is yet unknown and this was the focus of the present study.
70 Specifically, we investigated sequentially the up-take of IPNV from the intestinal lumen and its
71 subsequent distribution to lymphoid organs or to the liver of Atlantic salmon.

72

73 **2. Materials and Methods**

74 This study was approved by the Norwegian Animal Research Authority.

75 **2.1 Cell lines and viruses**

76 Asian grouper strain K (AGK) cells [15] were used for the propagation of virus in this study. The
77 cells were grown in L-15 medium (Invitrogen) supplemented by 7.5% fetal bovine serum as well
78 as 10% L-glutamine and were incubated at 28 °C. Chinook salmon embryo cells (CHSE-214)
79 were used for titration of the virus and were maintained at 20 °C in the same medium as AGK
80 cells but with 10% FBS. When infected with IPNV, only 1% FBS was used in the media of both
81 cell lines as well as 1 mg/ml of gentamicin. The incubation temperature was then set to 15 °C
82 until full CPE.

83 **2.2 Fish and rearing conditions**

84 Approximately 90 Atlantic salmon parr weighing about 25 g each were procured from Sørsmolt
85 AS in Sannidal, Norway. The fish were healthy and the hatchery from which they were purchased
86 had had no previous records of IPNV outbreaks in the three years prior to the study. The fish

were transported to the Norwegian University of Life Sciences/Veterinary Institute shared wetlab in Oslo by road in oxygenated bags. One week following acclimatization, the fish were treated with formalin (diluted 1:4000 in water) for 30 min against ectoparasites. The fish were then kept for a further week prior to the onset of the experiment. During the entire experiment the water temperature was 12 °C.

2.3 Antigen administration/infection of the fish

Infectious pancreatic necrosis virus grown in AGK cells to a titer of 10^9 $TCID_{50}$/ml as described above was used. Inactivation of the virus was done as described by Dixon & Hill (1983). Briefly, 0.5% formalin (w/v) was added to the virus supernatant followed by incubation at room temperature for 48 hrs with a magnetic stirrer. Formalin was then removed by dialysis against PBS. To test for inactivation and the presence of residual formalin, fresh CHSE cells were incubated with excessive amounts of inactivated virus supernatant. No CPE or cellular toxicity was observed after 7 days.

Prior to treatment of the fish, the feed was with-held for 24hrs. Allocation of the fish into 6 groups was done sequentially by dip netting. Prior to intubation, the fish were anaesthetized by using Benzocaine at 10mg/L of water. The virus was administered into the fish by using a 1ml syringe and tube. Treatment groups comprising 15 fish each were as follows: 1) Live IPNV administered orally; 2) Live IPNV administered anally; 3) Inactivated IPN administered orally; 4) Inactivated IPNV administered anally; 5) L-15 medium only administered orally; 6) L-15 medium only administered anally. Each fish received 0.3 ml of the preparation. Marking of the fish was by fin-clipping and each of the four groups (Live-oral; Live-anal; inactivated-oral;

Inactivated-anal) was kept in a separate tank. The controls (media only) were kept together with the inactivated virus groups.

2.4 Sampling

At 1, 24, and 72 hrs post intubation (h.p.i.), 5 fish from each group were sacrificed and sampled. Samples of blood, liver, spleen, kidney, anterior intestine or mid-gut (immediately caudal to the pyloric caeca) and posterior intestine or hindgut (1 cm cranial to the anus) were collected. Blood samples were centrifuged on site and then serum was aspirated and transferred to clean tubes for storage at -80 °C until required. The rest of the samples were preserved in 10% phosphate buffered formalin.

2.5 Enzyme-linked immunosorbent assay (ELISA)

In order to assess the amount of IPNV in the blood of the different groups of fish, 96-well plates were coated with 100 µl serum from experimental fish diluted at 1:40 in coating buffer (0.1 M Carbonate buffer pH9.6). The plates were then incubated at 4 °C overnight. The next morning, the plates were washed before blocking with 100 µl of 5% dry milk for 2 hrs at room temperature. Unless otherwise stated, all washing steps were done using 200 µl of PBST/well. Dry milk was diluted with PBST while antibodies were diluted with 1% dry milk. Following blocking, the plates were washed and then incubated with 100 µl of 1:1000 rabbit anti-IPNV antibodies (K95) [16] at room temperature for 1 hr. Following a washing step, 100 µl of secondary antibody, peroxidase-labelled goat anti rabbit (DAKO; Glostrup, Denmark) diluted at 1:1000 dilution, was incubated in each well at room temperature for 1 hr. After another washing step, 100 µl OPD substrate was added to each well and incubated at room temperature for 15 min. The reaction was

then stopped by adding 50 µl/well 1M H_2SO_4. OD values were detected by using an ELISA reader at 492 nm absorbance.

2.6 Immunohistochemistry

Staining of tissues was carried out as described by Evensen and Lorenzen 1996 [17]. Briefly, after de-paraffinization and rehydration, tissue sections were blocked with 5% BSA (Sigma Aldrich) diluted in 1 M Tris buffer solution (TBS) pH 7.6 for 30 min. Subsequently, 150 µl of rabbit anti-IPNV serum (K95) diluted at 1:1000 in 2.5% BSA was added to each slide. After incubating for 30 min at room temperature, the slides were washed. All washing steps were carried out using 1 M Tris-buffer pH 7.6 with 1% Tween 20. Biotinylated goat anti-rabbit antibody (DAKO; Glostrup, Denmark) was then added for 30 minutes. After washing, streptavidin alkaline phosphatase (Sigma-Aldrich) was added and incubated for 30 min. Following washing, fast red substrate (Sigma-Aldrich) was added to each slide and incubated for 10 min. The reaction was stopped by immersion of slides in running tap water for 5 min. Counterstaining was carried out using Hematoxylin dye for 2 min and then washing in tap water. After mounting with glycerol, the slides were observed under a light microscope.

2.7 Statistical analysis.

Differences in antigen scores as detected by ELISA between groups were analyzed by a two-tailed Student's t test assuming populations of equal variance. To analyze differences between treatment types (live versus inactivated) and routes of intubation (oral versus anal) on the response (antigen present/not present as detected by immunohistochemistry), Fisher's exact test was used with the help of the JMP® statistical software [18].

3. Results

At 1 hr following intubation with live virus, 2/5 fish in the anally intubated group and 3/5 fish in the orally intubated group died prematurely and were excluded from analysis. Therefore the numbers of samples collected at this time point were reduced accordingly. All five fish from each group were sampled from the rest of the time points.

3.1 Higher IPNV antigens were detected in orally- compared to anally-intubated fish by ELISA

No antigens were present in the control fish. The OD values for treatment exhibited three trends: 1) an increase in IPNV antigens in the serum of all groups from 1 to 24 hrs post intubation (h.p.i.) followed by a decrease; 2) Higher serum antigens ($p<0.04$) in groups intubated orally (live and inactivated) compared to those intubated anally (Fig. 1); 3) A higher trend of serum antigens in live groups compared to inactivated ones albeit non-significantly. Another notable contrast was in the group intubated anally with inactivated IPNV whose OD values were consistently low at all-time points, being comparable to the negative controls ($p=0.05$).

3.2 Detection of IPNV antigens in different tissues by immunohistochemistry

Table 1 shows the number of fish in which positive antigens were demonstrated by immunohistochemistry in different groups while Table 2 is a summary of the results by organ distribution. Significantly more antigens ($p<0.03$) were observed in fish intubated with inactivated virus compared to those with live virus when all tissues in each group were summed up.

The number of fish per group in which antigens were demonstrated is shown in Table 2. No antigens were detected from the anterior intestine of any of the groups. In contrast, antigens were observed in the posterior intestine of some of the fish intubated both orally and anally with inactivated IPNV from 1 hour post intubation (p.i.) onwards. In general, more antigens were observed in fish intubated with inactivated antigens than live virus ($p<0.03$) although the loss of some fish at the start of the experiment might have affected this result (Table 1). No difference between intubation routes was observed.

Antigens were observed in the posterior intestine of the inactivated virus groups (both anally and orally) at 1hr and 72 h.p.i. The antigens were located in the cytoplasm of enterocytes and macrophage-like cells (Fig. 3a).

In immune organs (head kidney and spleen), more antigens were observed in immune organs of fish intubated with inactivated antigens compared to those with live IPNV. The antigens were observed at all-time points and were localized in macrophages and melanomacrophages (Fig. 3b).

No IPNV antigens were observed in the livers of any of the fish groups.

4. Discussion

In the present study, the uptake of IPNV by enterocytes in the posterior intestine, the hematogenous distribution and localization in head kidney and spleen of Atlantic salmon were demonstrated. Both live and inactivated IPNV antigens were observed in the cytoplasm of enterocytes and macrophage-like cells as early as 1 hr post intubation. The antigens were also observed in the named organs at 72 h.p.i. To our knowledge, this is the first report to document

198 the uptake of this virus both live and inactivated from the lumen of the intestines. The
199 anticipation is that the mechanisms involved are similar to that of HRP or ferritin as reported by
200 others [7]. These findings support previous reports that the intestine is important for absorption of
201 macromolecules in fish [13]. Although no antigens were demonstrated in the anterior intestine in
202 the present study, the higher serum antigens in orally intubated groups compared to their anal
203 counterparts as assessed by ELISA suggest that the anterior intestine as well as the foregut may
204 be important in the uptake of IPNV as suggested by some [13] while contrasting others [7;13].
205
206 Orally administered antigens are believed to be depleted by the time they get to the posterior
207 intestine due to the negative actions of the stomach environment [7;13]. In the present study, anal
208 intubation of antigens was included to contrast the oral in order to test this effect. More antigens
209 were observed in fish intubated orally compared to anally ($p<0.04$) in contrast to the findings of
210 others [7;13]. These results suggest that IPNV is resistant to the low pH and digestive enzymes
211 found in Atlantic salmon stomach. This is hardly surprising given that IPNV is well known to
212 resist chemical and even thermal treatments [19-21].
213
214 The uptake of live IPNV was in general comparable to that of the inactivated virus administered
215 orally as measured either by ELISA or immunohistochemistry. This is despite the fact that the
216 live virus has the capacity to multiply and increase in the fish within the time frame of this study.
217 These findings are consistent with reports of others [22;23], offers support to receptor-mediated
218 uptake suggesting that formalin inactivation of IPNV does not significantly alter its surface
219 structure, thereby allowing the virus to be taken up as efficiently. When it comes to anally
220 administered inactivated IPNV however, it is noteworthy that the serum antigens were low at all-
221 time points.

In the present study, antigens of both live and inactivated virus intubated either orally or anally were observed in the head kidney and spleen of the fish from 1hr to 72 hrs. Antigens were localized in the cytoplasm of erythrocytes, macrophage-like cells as well as melanomacrophages. The presence of antigens in these cells is in line with previous reports of antigen retention in immune organs [24]. The headkidney and spleen of fish are antigen-trapping organs that filter out systemic antigens with melanomacrophage centres serving as focal repositories and may be primitive analogues of germinal centres of lymph nodes [25;26]. Melanomacrophage centres contain lymphocytes and are probably sites where immune activation of trapped antigens occurs [27].

No IPNV antigens were observed in the liver of any groups in the present study. These findings are in agreement with our previous work [28] but contrast the report of others [11]. The reason for this difference is likely methodological as immunohistochemistry were used in our studies while virus re-isolation from cellular fractions was used in the latter. Furthermore, the fish species and probably virus strains were also different. Since salmonid liver, unlike the spleen and kidney, receives mostly venous blood from the gut [29-31] and plays a role in the digestion and removal of toxins from the blood, one would expect that all antigens taken up by the intestine would be observed in this organ. This negative result therefore suggests that hepatocytes might not be readily susceptible to IPNV and this view auger well with previous reports that the liver is one of the last organs to be compromised following IPNV infection [28].

Acknowledgement

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Figure legend

Figure 1

Infectious pancreatic necrosis virus in the serum of different groups of Atlantic salmon at different time points following intubation. ELISA. *significantly different ($p<0.05$) from the control.

Figure 2

Contingency table analysis of the response of Atlantic salmon to administration of infectious pancreatic necrosis virus. A) Comparison between routes of administration and B) Comparison between virus treatments. Key: 0= no antigens detected; 1= antigen detected in the fish; Oral: n= 27; Anal: n= 28; Live virus: n= 25; Inactivated virus: n=30.

Figure 3

Infectious pancreatic necrosis virus antigens (red stain) in different tissues of Atlantic salmon at designated time points following oral or anal intubation. Immunohistochemistry. A) Posterior intestine, inactivated IPNV at 72 hrs post oral intubation; B) Head kidney, inactivated IPNV at 72 hrs post oral intubation; Key: E=enterocytes; MØ = macrophages; MMØ= melanomacrophages. X40 magnification.

Tables

Table 1.

Proportion of fish showing infectious pancreatic necrosis virus staining in different organs following intubation with either live or inactivated virus.

Treatment	Route of intubation	Time (hours post intubation)		
		1	24	72
Live virus	Oral	0/2	1/5	3/5
	Anal	3/3	0/5	0/5
Inactivated virus	Oral	4/5	1/5	1/5
	Anal	4/5	3/5	4/5

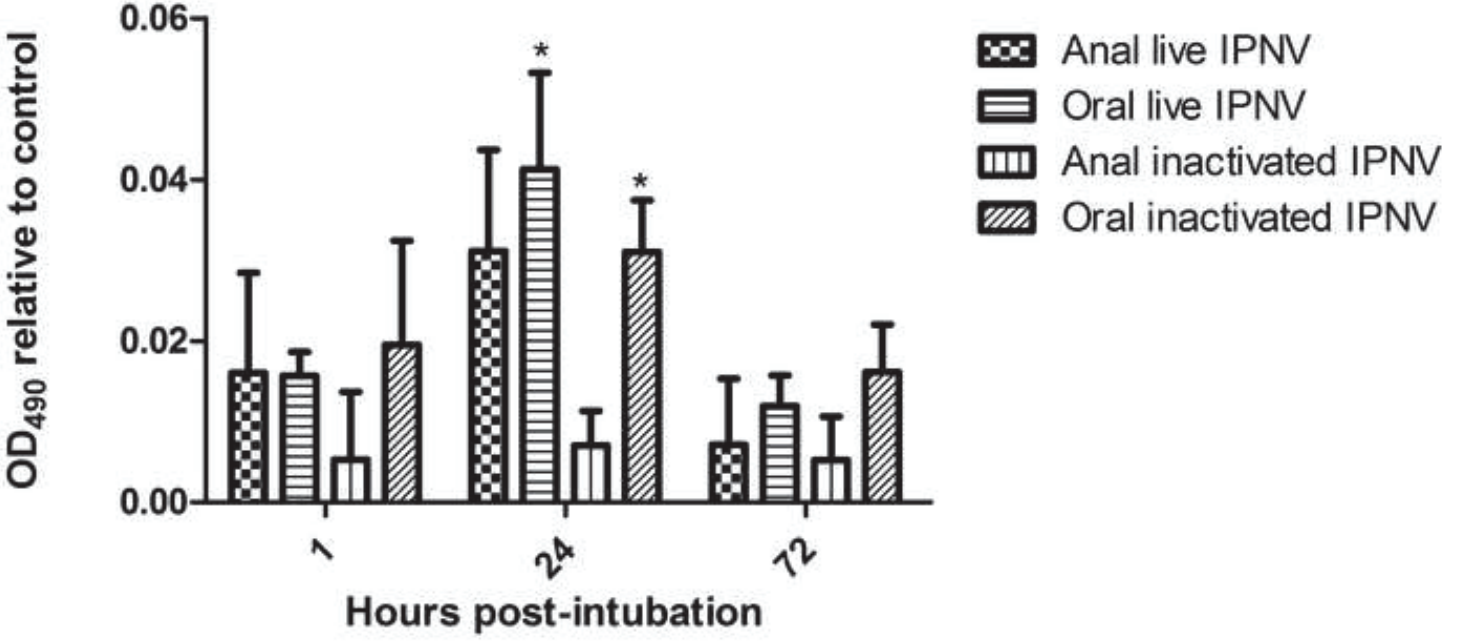
Table 2.

Number of fish with positive staining for infectious pancreatic necrosis virus antigens in different tissues following intubation.

Tissue	Time	Live virus		Inactivated virus	
	(hours p.i.)	Anal intubation	Oral intubation	Anal intubation	Oral intubation
Posterior intestine	1	0*	0**	1	1
	24	0	0	0	0
	72	0	0	1	1
Liver	1	0*	0**	0	0
	24	0	0	0	0
	72	0	0	0	0
Head kidney	1	3*	0**	2	3
	24	0	1	2	0
	72	0	3	3	0
Spleen	1	2*	0**	1	2
	24	0	0	3	1
	72	0	0	2	0

*n=3; **n=2 otherwise n=5.

Figures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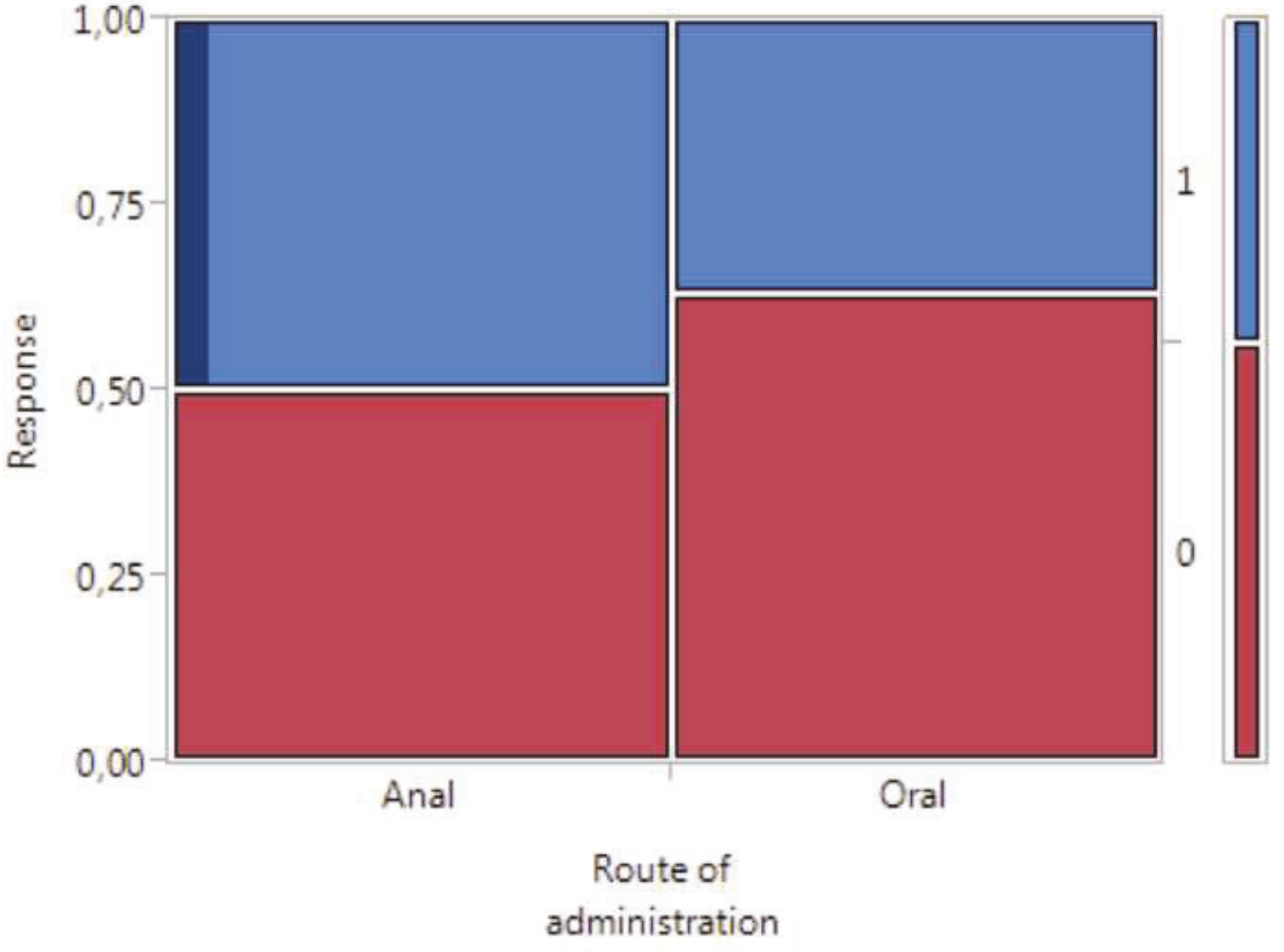
Figures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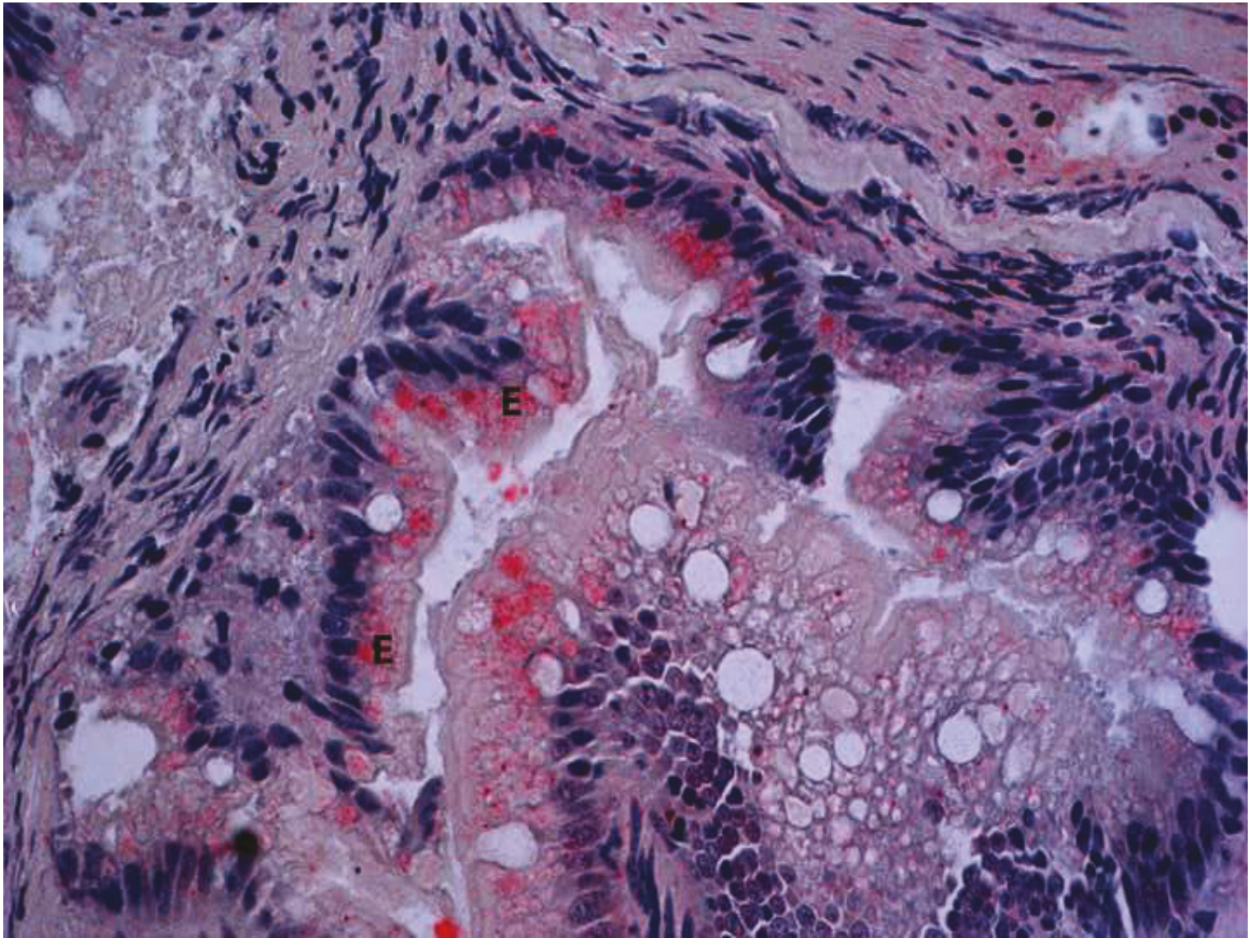
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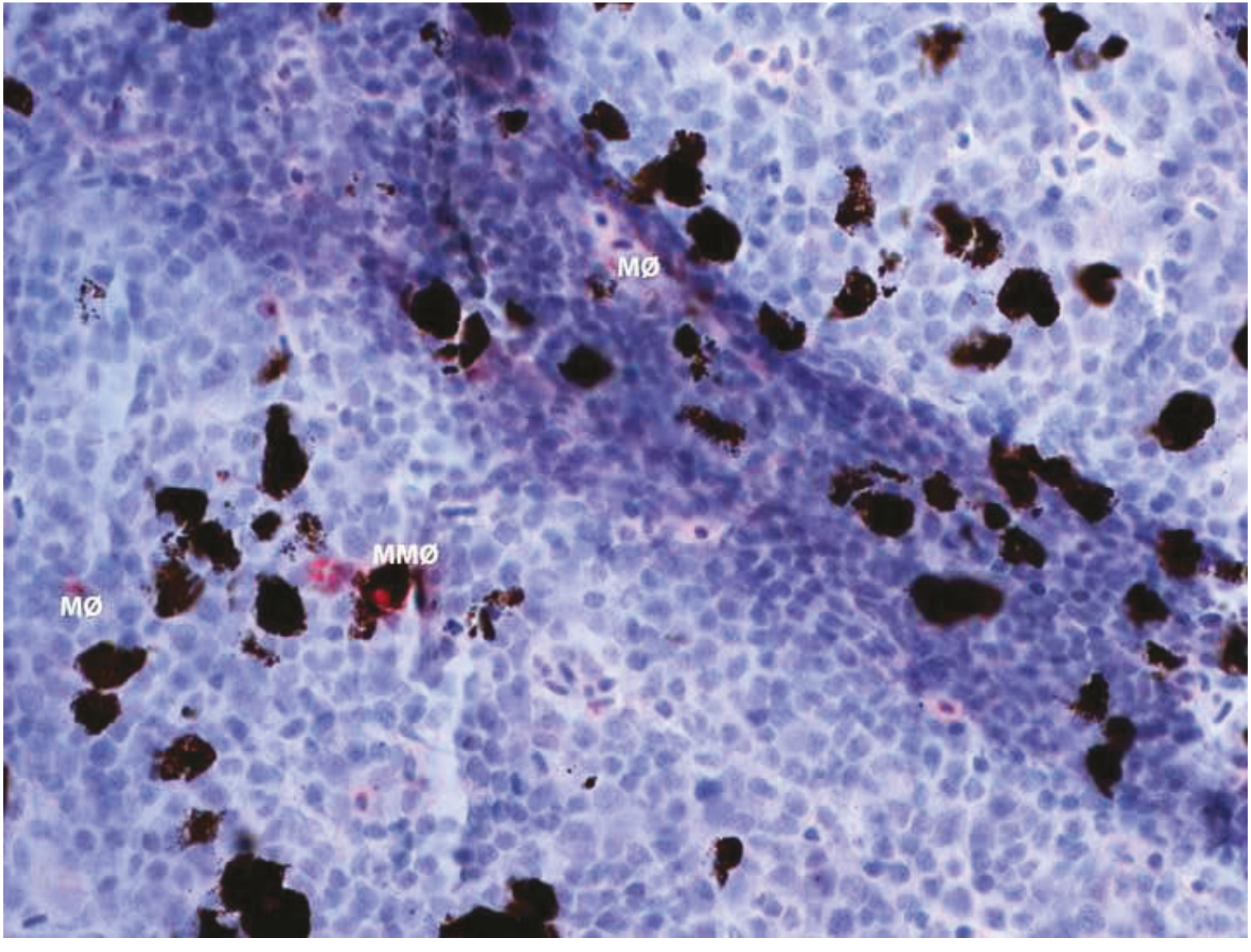
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III

Augmentation of the antibody response of Atlantic salmon by oral administration of alginate-encapsulated IPNV antigens

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Abstract

The objective of the present study was to assess the effect of alginate-encapsulated infectious pancreatic necrosis virus antigens in inducing the immune response of Atlantic salmon as booster vaccines. One year after intraperitoneal injection with an oil-adjuvanted vaccine, post-smolts were orally boosted either by 1) alginate-encapsulated IPNV antigens (ENCAP); 2) soluble antigens (UNENCAP) or 3) untreated feed (control). This was done twice, seven weeks apart. Sampling was done twice, firstly at 7 weeks post 1st oral boost and the 2nd, at 4 weeks after the 2nd oral boost. Samples included serum, head kidney, spleen and hindgut. Serum antibodies

were analyzed by ELISA while tissues were used to assess the expression of IgM, IgT, CD4, GATA3, FOXP3, TGF-β and IL-10 genes by quantitative PCR. Compared to controls, fish fed with ENCAP had a significant increase ($p<0.04$) in serum antibodies following the 1st boost but not after the 2nd boost. This coincided with significant up-regulation of CD4 and GATA3 genes. In contrast, serum antibodies in the UNENCAP group decreased both after the 1st and 2nd oral boosts. This was associated with significant up-regulation of FOXP3, TGF-β and IL-10 genes. The expression of IgT was not induced in the hindgut after the 1st oral boost but was significantly up-regulated following the 2nd one. CD4 and GATA3 mRNA expressions exhibited a similar pattern to IgT in the hindgut. IgM mRNA expression on the other hand was not differentially regulated at any of the times examined. Our findings suggest that 1) Parenteral prime with oil-adjuvanted vaccines followed by oral boost with ENCAP results in augmentation of the systemic immune response; 2) Symmetrical prime and boost (mucosal) with ENCAP results in augmentation of mucosal immune response and 3) Symmetrical priming and boosting (mucosal) with soluble antigens results in the induction of systemic immune tolerance.

Keywords: Oral vaccine, IPNV, alginate, boost, immune response

1. Introduction

Infectious pancreatic necrosis is an important disease of salmonids responsible for great economic losses in the aquaculture industry. It is characterized by loss of appetite, darkened skin pigmentation, distended abdomen and mortalities ranging from negligible to almost 100%. Histopathologically, necrosis of pancreatic acinar cells, multifocal hepatic necrosis and acute catarrhal enteritis are commonly observed [1,2]. The causative agent is infectious pancreatic necrosis virus (IPNV), a double stranded RNA virus belonging to the family *Birnaviridae* and genus *Aquabirnavirus* where it is the type species.

Control of IPN is by vaccination and oil-based vaccines have earned their place in the market mainly because of their contribution to the control of bacterial diseases in the late 80s and early 90s in Norway. The efficacy of these vaccines against diseases caused by intracellular pathogens such as viruses however remains equivocal, thus the need for the continued search for more effective vaccines.

The most desirable vaccines for higher vertebrates and even more so for fish are those delivered orally because of the ease with which they are administered; are stress-free; applicable to smaller fish and are less labour-intensive [3]. Their usage in the aquaculture industry has however been under-exploited because of their poor performance in comparison with injectable and immersion counterparts. Some of the challenges associated with orally delivered vaccines include poor antigen delivery and uptake, degradation during passage through the digestive tract and induction of tolerance [4,5]. Nevertheless, a report of good protection in fish vaccinated with encapsulated DNA plasmids has recently been published [6]. Unfortunately,

legislation in most countries at the moment precludes the use of DNA vaccines in food animals [7,8].

One of the challenges faced by vaccination of fish is the duration of protection conferred by different preparations. As already mentioned, oil-based vaccines induce long lasting protection against several bacterial pathogens but this could be at the cost of severe side effects [9]. For viral diseases including IPN, most products on the market do not give satisfactory protection probably because of their failure to induce sufficiently high antibody titers required prior to challenge [10]. Boosting is a good alternative for enhancing or extending protection as shown for lactococcosis [11]. The effect of boosting against IPNV in particular and oral vaccination in general is however not well understood. The main purpose of the present study therefore was to assess the effect of alginate-encapsulated IPNV in stimulating the immune system of Atlantic salmon as a booster vaccine.

2.0 Results

2.1 Intake of oral boost feeds and IPNV antigen dose

The average weight of the fish during the primary and secondary oral boost feeding, the feed intake and antigen dose are shown in Table 1. As targeted, the average antigen dose administered during each of the boost periods was about 1×10^9 $TCID_{50}$/fish. However, due to the doubling in the fish weight between the two boost periods, the dose per kg of fish body weight during the second boosting was almost half that during the first (Table 1).

93 **2.2 Antigen retention in head kidneys and hindguts**

94 To estimate the amount of antigen taken up and retained both locally and systemically
95 in each group, qPCR was used targeting hindgut and head kidneys tissues to examine
96 retained antigens at the time of sampling (7 weeks and 4 weeks following the 1st and
97 2nd boosts, respectively). The head kidney was used to represent the systemic
98 compartment since this is one of the main antigen trapping organ for blood-borne
99 antigens in fish [12].

100

101 The results show that more mRNA of IPNV (used as a surrogate of antigens) were
102 retained in the encapsulated (ENCAP) versus unencapsulated (UNENCAP) groups at
103 both time points (Fig. 1). In the head kidney, a significant increase in antigens
104 retained from the 1st to the 2nd sampling was observed in both groups. A similar trend
105 in the ENCAP group was observed in hindgut while for the UNENCAP group, no
106 difference between sampling times was observed (Fig. 1).

107

108 **2.3 Oral boosting with alginate-based antigens induces a systemic but transient**
109 **IgM antibody response**

110 Since all experimental fish had previously been vaccinated with an oil-based vaccine
111 that contained IPNV antigens, all the fish had relatively high specific background
112 antibodies as expected. The un-boosted group (control) was used as the baseline for
113 comparison with boosted groups.

114

115 In general, the response of antibodies in boosted groups showed a reverse trend over
116 time compared to that of antigens (Fig. 1&2). In the group boosted with ENCAP, the
117 antibody response was significantly higher ($p<0.04$) than the control following the 1st

boost (Fig. 2). At 4 weeks following the 2nd boost however, the antibodies had returned to background levels. In the UNENCAP group, no difference was observed compared to unboosted controls during the 1st sampling while the antibodies were significantly suppressed ($p<0.02$) following the 2nd boost (Fig. 2).

2.4 The systemic immune response is predominantly Th2

The induction of antibodies in the ENCAP group was suggestive of a predominantly humoral response. Thus to verify this, we examined the expression of CD4 and GATA-3 genes that are known to be associated with Th2 responses, in the head kidney and spleen.

At both 7 weeks post primary- and 4 weeks post-secondary boost, the ENCAP group had significantly higher CD4 expression ($p<0.04$) in both the head kidney and spleen compared to other groups (Fig. 3a). In contrast, this gene was suppressed in the UNENCAP group at the 1st sampling albeit non-significantly. At the 2nd sampling, this gene was significantly suppressed ($p<0.01$) in this group. In the ENCAP group, the expression of GATA3 was significantly up-regulated ($p<0.04$) at both time points in the head kidney and spleen, consistent with the results of CD4 while in the UNENCAP group, these genes were not induced (Fig. 3b).

2.5 The gut mucosal response is also Th2 and is primarily primed by the oral route

To assess the gut mucosal response, we examined the expression of IgT mRNA since antibodies are not available to us. In addition, mRNA expression of IgM, CD4 and GATA3 genes were also assessed.

After the 1st oral boost, marginal but non-significant inductions of IgT were observed in both the ENCAP and UNENCAP groups compared to the control (Fig 3c). At 4 weeks following the 2nd boost, the expression increased in both groups but more significantly ($p<0.01$) in the ENCAP compared to UNENCAP group. Interestingly, CD4 and GATA3 expression in this organ had a similar pattern to IgT (Fig. 3a&b), especially for the ENCAP group. Conversely, CD4 and GATA3 expression of the UNENCAP group were generally not induced at both time points. The expression of IgM in the hindgut was not differentially expressed in all groups at all-time points examined (Fig. 3d).

The assessment of IgT expression was extended to the head kidney and spleen where at all-time points and in all groups, this gene was not differentially expressed. The only exception was in the head kidney of the ENCAP group, at 4 weeks following 2nd boost (Fig. 3c), where it was significantly up-regulated ($p<0.03$).

2.6 Repeated oral administration of IPNV antigens results in decreased serum antibodies but not in the hindgut of Atlantic salmon

The reduction in serum antibodies following administration of antigens can be due to consumption [10,13]. Thus in order to check whether this was the case in the present study, we examined the transcript levels of IgM. Fig. 3d shows significant down-regulation of B cell IgM transcripts both in the kidneys ($p<0.01$) and spleen (0.001) of the UNENCAP group from the 1st to the 2nd samplings. A similar change was observed in the spleen of the ENCAP group ($p<0.01$) but without any differential regulation in the kidney.

In order to gain insight into the suppression of antibody production by B cells in the UNENCAP group, we examined the expression of forkhead box protein 3 (FOXP3), TGF-β and IL-10.

Consistent with the reduction/suppression of antibodies in the UNENCAP group at 4 weeks following 2nd boost, the expression of FOXP3, TGF-β and IL-10 were all significantly up-regulated ($p<0.003$, 0.035 and 0.0048, respectively) in the kidneys of this group (Fig. 4a-c). In contrast, no differential expressions of either of these genes were observed in any of the groups after the 1st boost or in the spleen.

In the hindgut, FOXP3 was only induced in the UNENCAP group after the 2nd boost (Fig. 4a) while both TGF-β and IL-10 were significantly induced in both ENCAP and UNENCAP groups ($p<0.02$ and 0.01 for TGF-β and $p<0.01$ and 0.01 for IL-10, respectively).

3. Discussion

The findings of the present study demonstrate that oral boosting of Atlantic salmon following parenteral injection with alginate encapsulated IPNV antigens induces both systemic and mucosal (gut) humoral responses. The significant up-regulation of CD4 ($p<0.02$) and GATA-3 ($p<0.04$) genes in the head kidneys and spleens of the ENCAP group (Fig. 3) fit very well with the induction of serum antibodies and point towards a predominantly T-helper 2 (T_H2) response. In higher vertebrates, the intestinal mucosa contains high basal levels of IL-4, IL-10 and TGFβ that are induced shortly after oral vaccination [14]. This micro-environment is thought to tip responses of oral vaccines towards T_H2 [4]. Furthermore, the main mechanism of action of alginate encapsulated

antigens has been proposed to be biased towards T_H2 responses [15–18]. These findings are consistent with reports of others using different antigens [19,20] and suggest that oral boosting with alginate encapsulated antigens holds promise as a means of augmenting immune responses against IPN. One limitation of this study is that the fish were not challenged following vaccination and therefore the protective effects of the responses could not be tested but should be a subject for future studies.

There are conflicting reports when it comes to protection against disease using orally administered vaccines, with some reporting success [6,11,20] while others found little or no difference from controls [17]. Several reasons can be attributed to these variations including the nature of antigens used, formulation of oral preparations, immune response generated versus that desired etc. In the present study, the fish were not challenged following vaccination owing to logistical constraints. While this should be a subject for further studies, it is known from a previous study that high antibody titers against IPNV at the onset of challenge correlate with protection of the fish [10]. It is not unlikely therefore that the augmentation of the immune response in the present study may have been associated with protection.

It has previously been reported that oral vaccination results in transient antibody response lasting typically 3 weeks post vaccination [20–22], and most of the studies address parenteral/oral combinations or vice-versa. However, very few studies have examined the effect of repeated oral vaccination. In the present study, reductions in serum antibodies following two oral vaccination (7 weeks apart) was observed in the both the ENCAP and UNENCAP groups after the second oral boost. This is consistent with the findings of others who used a similar administration regime (5

219 days of oral vaccine administration per month) [17]. In the same study however,
220 administering the oral vaccine at a rate of 3 days/week for 2 months resulted in
221 progressive increase in antibody titers over time. Together, these findings suggest that
222 modality by which oral vaccines are administered can determine whether a booster
223 effect or tolerance ensues. The low CD4 expression in the UNENCAP group in the
224 kidney and also its decline in the ENCAP group concurrent with the lack of induction
225 of GATA3 and IgM expression in the present study is however intriguing. It is
226 tempting to speculate that this could point towards anergy as discussed further below.
227
228 The induction of the systemic response as measured from serum antibodies and
229 immune gene expression in the kidney of the ENCAP group following the 1st oral
230 boost is in line with previous reports [20,22]. The fact the 1st oral boost did not induce
231 a corresponding change in the mucosal response as measured by gene expression in
232 the hindgut suggests that injection vaccination does not activate mucosal immunity, in
233 common with findings of others [20]. Furthermore, these results (Fig. 3a-c) suggest
234 that in this study, the 1st oral boost served as a "prime" to the mucosal response while
235 the 2nd one "boosted" it. Interestingly, oral boosting had no effect on the IgM
236 expression in the hindgut, a difference from what others have observed assessing
237 mucosal antibodies [20]. The ability of orally administered antigens to stimulate both
238 systemic and mucosal immune responses on one hand and parenteral vaccination
239 inducing only a systemic response demonstrate asymmetrical responses of immune
240 induction as previously observed in mice [23].
241
242 One of the challenges of oral vaccination in fish is the induction of tolerance. This has
243 been shown to be easily induced with soluble antigens [21]. In the present study,

tolerance was induced by two booster administration of encapsulated IPNV antigen (UNENCAP) feeds (Fig. 2). In higher vertebrates, tolerance is the default immune pathway in mucosal surfaces and is related to the dose of antigens given, i.e. high doses lead to anergy/deletion while the opposite leads to regulatory T cells (Treg) induction [24]. The suppression of antibodies in the present study coupled with the induction of FoxP3, TGF-β and IL-10 (Fig. 4) suggests the involvement of both mechanisms. In higher vertebrates, FOXP3 is a key transcription factor of regulatory T cells (Tregs) while TGF-β is known to induce T cells including Tregs [24,25]. IL-10 on the other hand is an anti-inflammatory cytokine that has been shown to contribute towards the induction of immune tolerance [24]. These genes have also been described in fish although their functions relative to immune tolerance remain to be characterized.

While the induction of tolerance may be testimony that much of the un-encapsulated IPNV antigens were taken up, meaning they survived the hostile acidic environment in the stomach, this finding underlines the importance of encapsulation as an aid to stimulating the immune response of fish. It is noteworthy that the doses of vaccines administered during the 2nd boost were lower per body weight of fish compared to the 1st boost since the fish had gained weight by the time they received the second boost. The effect of this was not addressed but should be a subject of future studies.

Finally, the findings of the present study can be summarized as follows**:** 1) Parenteral prime with oil-adjuvanted vaccine followed by oral boost with ENCAP results in a the augmentation of both the systemic and mucosal immune responses; 2) Mucosal (gut) immunity is primarily primed by oral administration of antigens; 3) Oral prime and boost with ENCAP results in transient augmentation of mucosal immune responses

and 4) Oral priming and boosting with UNENCAP results in the induction of tolerance.

4.0 Materials and Methods

This study was approved by the Norwegian Animal Research Authority. Prior to sampling, the fish was anaesthetised with Finquel® (Scanvacc) at 100 mg/L in order to prevent suffering.

4.1 Cell culture

Asian grouper strain K (AGK) cells and Chinook salmon embryo cells (CHSE-214; ATCC CRL-1681) [26] were maintained with L-15 medium (Invitrogen) supplemented with 10% L-glutamine and 1µl/ml of gentamicin. In addition the medium used with the former also contained 7.5% fetal bovine serum (FBS) and these cells were kept at 28°C while the medium for CHSE cells contained 10% FBS and the cells were maintained at 20 °C.

4.2 Fish

The experiment was conducted at EWOS Innovation AS facilities in Dirdal, Norway. Healthy Atlantic salmon growers reared in sea water were used. The fish had been vaccinated with ALPHA JECT micro® 6 (PHARMAQ) about a year prior to the first boost treatment and were kept at a water temperature of 12°C throughout the experimental duration.

4.3 Vaccine preparations

4.3.1 Antigen preparation

A recombinant Sp strain of IPNV (rNVI-15PTA) [10] was used and was prepared as reported previously (Chen et al., 2013). Briefly, approximately 80% confluent AGK cells maintained in L15 media as described above but with 2% FBS were inoculated

297 with IPNV using MOI=0.1 followed by incubation at 15° C. Note: CHSE or RTG-2
298 cells can be used as alternatives in the absence of AGK cells, with post-culturing
299 concentration to increase virus amounts if necessary. The virus was harvested
300 following full CPE by centrifugation of the suspension at 2500 x g followed by
301 recovery of the supernatant. Titration of the virus was by end point dilution and the
302 titer measured using the Spearman–Karber's 50% tissue culture infectious dose
303 ($TCID_{50}$) in CHSE-214 cells.
304
305 The virus was inactivated with formalin (0.5% final concentration equal to 0.2%
306 formaldehyde) at room temperature for 48 hours with continuous stirring using a
307 magnetic stirrer. Thereafter formalin was removed by dialysis. Inactivation was
308 confirmed by inoculating confluent CHSE-214 cells while formalin residual effects
309 were tested by incubating cells with excessive inactivated virus and assessing for
310 toxicity.
311
312 **4.3.2 Antigen encapsulation and feed preparation**
313 The treatment groups of this study comprised either of the following feeding regimes:
314 1) untreated feed (control); 2) feed containing unencapsulated IPNV (in suspension);
315 3) feed containing alginate-encapsulated IPNV antigens.
316
317 Oral boost feeds (OBFs) were prepared by applying an oil mixture (OM) to Ewos
318 Opal 200 base pellet (BP) in a vacuum infusion coating process. OMs were
319 formulated by mixing IPNV Ag suspension (10^9 $TCID_{50}$/g), phosphate buffered saline
320 (PBS), (2.70×10^{10} $TCID_{50}$/g) with fish oil. Mixing was performed by using a
321 high-performance disperser (Model T25, IKA® Werke GmbH & Co., Germany) at

ambient temperature. The OBF were composed with the aim of generating feeds with an antigen level of 4.01×10^7 $TCID_{50}$/g. This level was selected due to an expected daily feed intake of 3.56g/fish during the first oral boost period. For the same reason, the targeted antigen level in the second OBF was 2.99×10^7 $TCID_{50}$/g. Control feeds were produced by mixing PBS with fish oil in advance of applying to BP in the vacuum infusion coating process.

4.4 Trial design and oral boosting

As part of a larger study examining responses of Atlantic salmon to different alginate formulations, 360 healthy Atlantic salmon weighing approximately 200g each were distributed by dip netting and sequential allocation into 9 circular 500L tanks containing sea water 10 weeks prior to the start of the primary oral boost. A description of the fish is given in section 4.2. The tanks were randomly divided into three groups (Unencap, Encap, and Control), with three tanks being assigned to each group (Figure 5).

The fish were fed with Ewos Opal 200 diet for 10 weeks prior to the first boost and also until the second boost. All fish groups were fed ad-libitum. During primary boosting, normal feed was replaced by OBFs for 7 days. After 7 weeks, the fish were sampled. Sampling was performed on 10 fish from each tank by first anaesthetizing the fish with Finquel® (Argent Laboratories) at 100mg/L. The following samples were collected, blood in heparin tubes for serum extraction; head kidney, spleen and hind gut in both RNAlater® (Invitrogen) and formalin.

Following sampling, a secondary boost was performed as described above. From then on, the fish were fed with Ewos Opal 500. The second sampling was done 4 weeks after the secondary boost.

4.5 Feed intake assessment

Unconsumed feed was collected during both boost periods to calculate feed intake. Uneaten pellets were spilled out of the tanks within 10 min post feeding and filtered off from the outlet water using an automatic collection system. Residual pellets were removed from the filters and put into a drying cabinet for 24 h at 70 °C. Amount of feed consumed was calculated as the difference between the dry weight of the feed served and the dry weight of unconsumed feed, expressed as the mass of feed per week per fish.

4.7 Enzyme-linked immunosorbent assay (ELISA)

Blood samples were centrifuged at 2500 x g for 10 min immediately after sampling. Thereafter, the serum was aspirated and transferred to new tubes that was the kept at -80°C until required.

ELISA was done as previously described [26] with minor modifications. Briefly, the wells of ELISA plates (Immunoplates, Nunc Maxisorb, Denmark) were coated with 100µl of polyclonal anti-IPNV [27] diluted 1:2000 in coating buffer (0.1M Carbonate buffer pH9.6) and then incubated at 4 °C overnight. The plates were washed prior to the incubation of 200µl of 5% dry milk per well for 2 hrs at room temperature. All washing steps were done in triplicate with 200µl PBST/well, all dilutions were with 1% dry milk and all incubation was at room temperatures unless otherwise stated.

After washing, the wells were incubated with 100µl of IPNV supernatant (10^8 $TCID_{50}$/ml) for 2 hours. Following another washing step, serum samples diluted 1:40 were then added to the wells and then incubated at 4 °C overnight. After washing, 100µl of mouse antibody against rainbow trout IgM [28] diluted in 1:5000 was incubated for 2 hours. Following another wash, 100µl of a 1:1000 dilution of peroxydase conjugated anti-mouse Ig (DAKO, Denmark) was incubated in each well for 1 hour. 100µl of OPD substrate (O-phenylenediamine dihydrochloride, DAKO) diluted in water was added to each well after washing. This reaction was incubated for 15 min following which the reaction was stopped by the addition of 50µl/well 1M H_2SO_4. Results were analyzed by using an ELISA reader (TECAN, Genios) at 492nm.

4.8 RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated by using the RNeasy Plus minikit (Qiagen) according to the manufacturer's instructions, and the concentration of RNA was determined by using the Nanodrop ND1000 (NanoDrop Technologies).

Quantitative PCR was performed by using QuantiFast SYBR Green RT-PCR Kit (Qiagen) and the LightCycler 480 system (Roche). For each gene, 50 ng of RNA was used as a template in a mixture of specific primers (250 µM) (Table 2) and QuantiFast SYBR Green RT-PCR master mix in a total volume of 20 µl. The mixtures were first incubated at 50°C for 10 min, then 95°C for 5 min, followed by 40 amplification cycles (10 s at 95°C; 30 s at 60°C and 8s at 72°C). The sequences of primers used are given in Table 2.

The $2^{-\Delta\Delta CT}$ method was used to calculate the gene products as described elsewhere [29] and is the relative mRNA expression representing the fold induction over the control group. All quantifications were normalized to β-actin.

4.9 Statistical analysis

The amount of feed intake for the three groups, gene expression and Elisa results were analyzed using Student's t test. F test was used to determine if the variances of population were equal or not. The threshold for significance was $p<0.05$ for both Student's t test and F test.

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510 **Figures Legends**

511 Figure 1. Infectious pancreatic necrosis virus (IPNV) mRNA expressed by real time
512 RT-PCR in selected organs of Atlantic salmon following oral boost with different
513 antigen preparations. This assay was used as a surrogate marker of retained
514 formalin-inactivated IPNV antigens in the present study. All fish were vaccinated
515 with an oil-based vaccine one year prior to the start of this study. The control fish
516 received no booster oral antigens. n=30; *statistically significant $p< 0.05$.

517

518 Figure 2. Infectious pancreatic necrosis virus-specific serum antibody levels in orally
519 boosted groups of Atlantic salmon relative to un-boosted controls. Antibodies were
520 assessed by ELISA and the results were obtained by using a plate reader (TECAN,
521 Genios) at a wavelength of 492nm. n=30; *statistically significant $p< 0.05$.

522

523 Figure 3. Mean relative expression of A) CD4; B) GATA3; C) IgT; and D) IgM genes
524 of fish orally boosted with different antigen preparations compared to un-boosted
525 controls. Real-time RT-PCR. n=30; *statistically significant $p< 0.05$.

526

527 Figure 4. Mean relative expression of A) FOXP3; B) TGF-β and C) IL-10 genes in
528 fish orally boosted with different antigen preparations compared to un-boosted
529 controls. Real-time RT-PCR. n=30; *statistically significant $p< 0.05$.

530

531 Figure 5. Schematic illustration of the trial plan used in the present study. Atlantic
532 salmon growers previously (1 year) vaccinated against IPNV were split into three
533 groups. Each group was further divided into three replicates (tanks) that were boosted
534 twice orally for one week. Sampling was at 7 weeks post primary boost (a day before

the second boost) and at the end of the trial. Ten fish from each tank (30 fish per group) were sampled at each time point.

TABLES

Table 1: Fish size in unit of mass (g), Weekly feed intake (FI) per fish, weekly IPNV antigen dose per fish and weekly IPNV antigen (Ag) dose per unit of fish mass (dose/kg).

Period	Group	Feed	Fish size (g) ±SD	Feed intake (g/fish/week) ±SD	IPNV Ag dose ($TCID_{50}$/fish/week) ±SD	IPNV Ag dose ($TCID_{50}$/kg/week) ±SD
Primary boost	Control	CF-1	395±92	24.7±0.4	0.00	0.00
	Unencap	OBF-1	375±82	23.9±2.4	$9.6\times10^{8}\pm1\times10^{8}$	$2.6\times10^{9}\pm6\times10^{8}$
	Encap	OBF-2	426±121	23.1±0.9	$9.3\times10^{8}\pm4\times10^{7}$	$2.2\times10^{9}\pm6\times10^{8}$
Second boost	Control	CF-2	846±135	39.6±0.4	0.00	0.00
	Unencap	OBF-4	796±126	37.3±3.9	$1.1\times10^{9}\pm1\times10^{8}$	$1.4\times10^{9}\pm3\times10^{8}$
	Encap	OBF-5	782±105	38.0±1.3	$1.1\times10^{9}\pm4\times10^{7}$	$1.5\times10^{9}\pm2\times10^{8}$

545 Table 2. Sequences of primers used in this study.

Genes	Accession number	Primer sequence 5'-3'	D*
β-actin	BT047241.2	CCAGTCCTGCTCACTGAGGC	F
		GGTCTCAAACATGATCTGGGTCA	R
IgT	HQ379938.1	AGAGGTGAAGACACACCGGTCATT	F
		ACGGAGTAGTTGCCTTTCTGGGTT	R
CD4	DQ867019.1	GAGTACACCTGCGCTGTGGAAT	F
		GGTTGACCTCCTGACCTACAAAGG	R
GATA3	NM001171800.1	CCCAAGCGACGACTGTCT	F
		TCGTTTGACAGTTTGCACATGATG	R
IgM	AF228580.1	TGAGGAGAACTGTGGGCTACACT	F
		TGTTAATGACCACTGAATGTGCAT	R
FOXP3	HQ270469	AGCTGGCACAGCAGGAGTAT	F
		CGGGACAAGATCTGGGAGTA	R
TGF-β	BT059581.1	AGTTGCCTTGTGATTGTGGGA	F
		CTCTTCAGTAGTGGTTTGTCG	R
IL-10	AB118099.1	CGCTATGGACAGCATCCT	F
		AAGTGGTTGTTCTGCGTT	R
IPNV	NC_001915.1	ATGCCAAGATGATCCTGTCCCACA	F
		TGCCTTTGAGGTTGGTAGGTCACT	R

D* direction of primer.

Figure 1

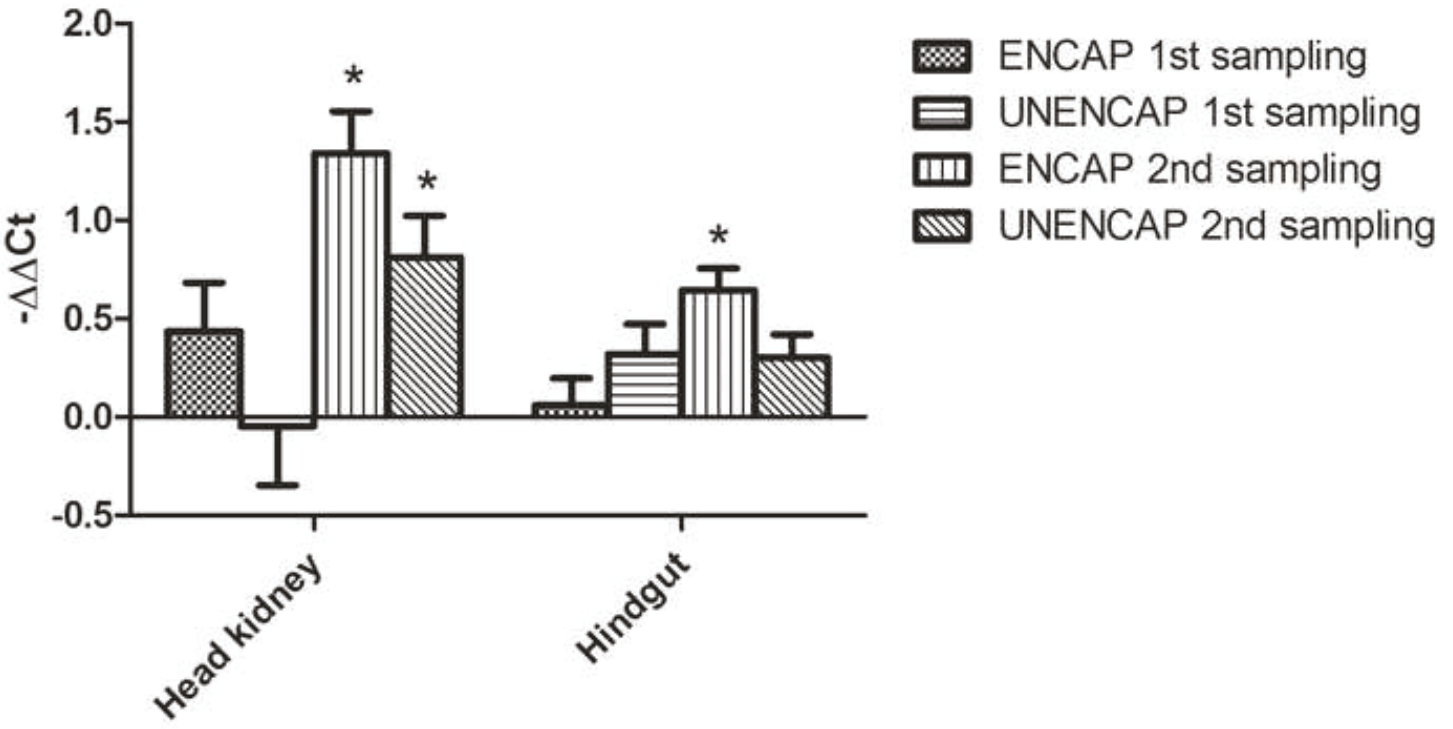

2.0
1.5
1.0
0.5
0.0
-0.5
-ΔΔCt
*
*
*
Head kidney
Hindgut
ENCAP 1st sampling
UNENCAP 1st sampling
ENCAP 2nd sampling
UNENCAP 2nd sampling

Figure 2

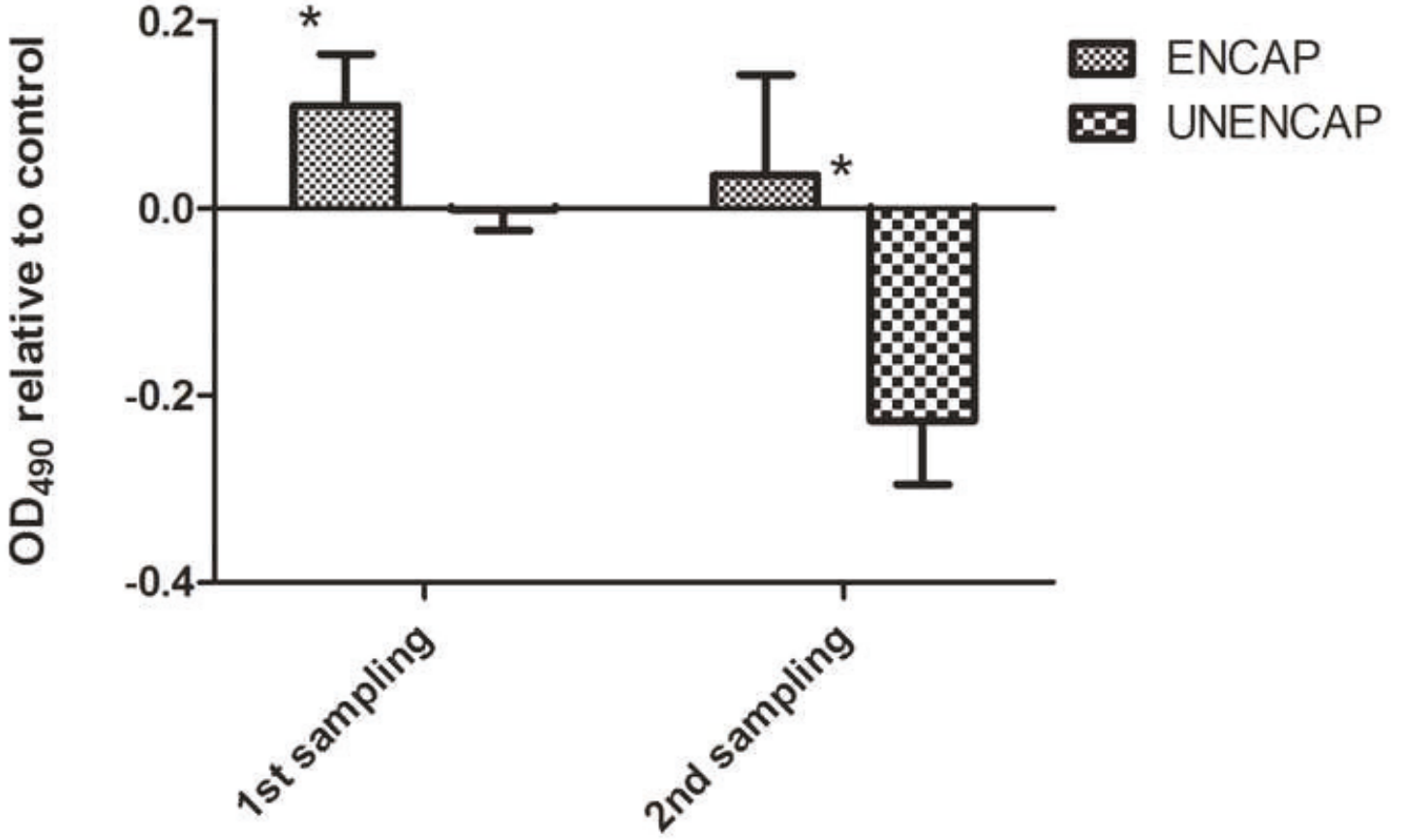

0.2
0.0
-0.2
-0.4
OD490 relative to control
*
*
ENCAP
UNENCAP
1st sampling
2nd sampling

Figure 3a

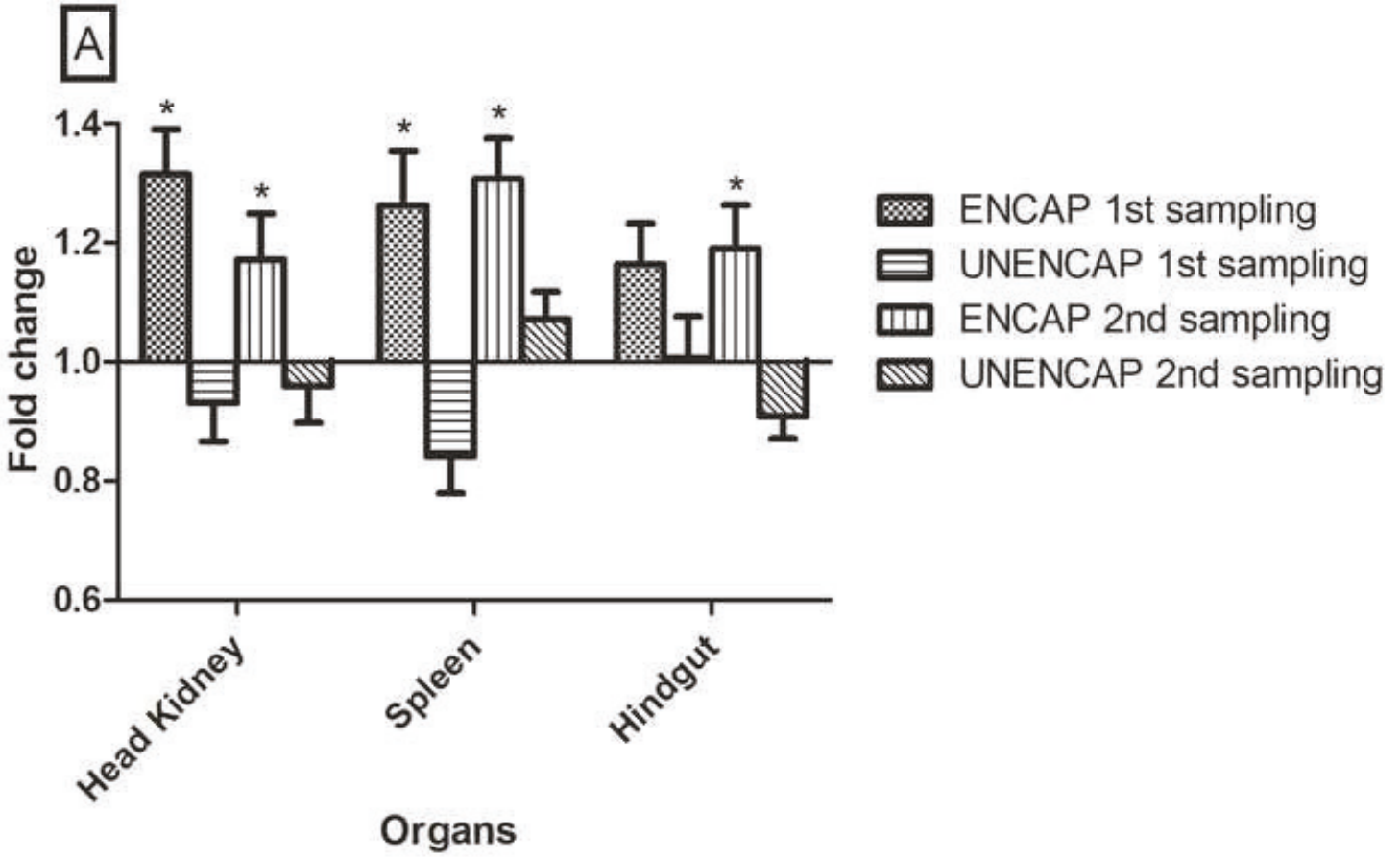

A
1.4
1.2
1.0
0.8
0.6
Fold change
*
*
*
*
*
Head Kidney
Spleen
Hindgut
Organs
ENCAP 1st sampling
UNENCAP 1st sampling
ENCAP 2nd sampling
UNENCAP 2nd sampling

Figure 3b

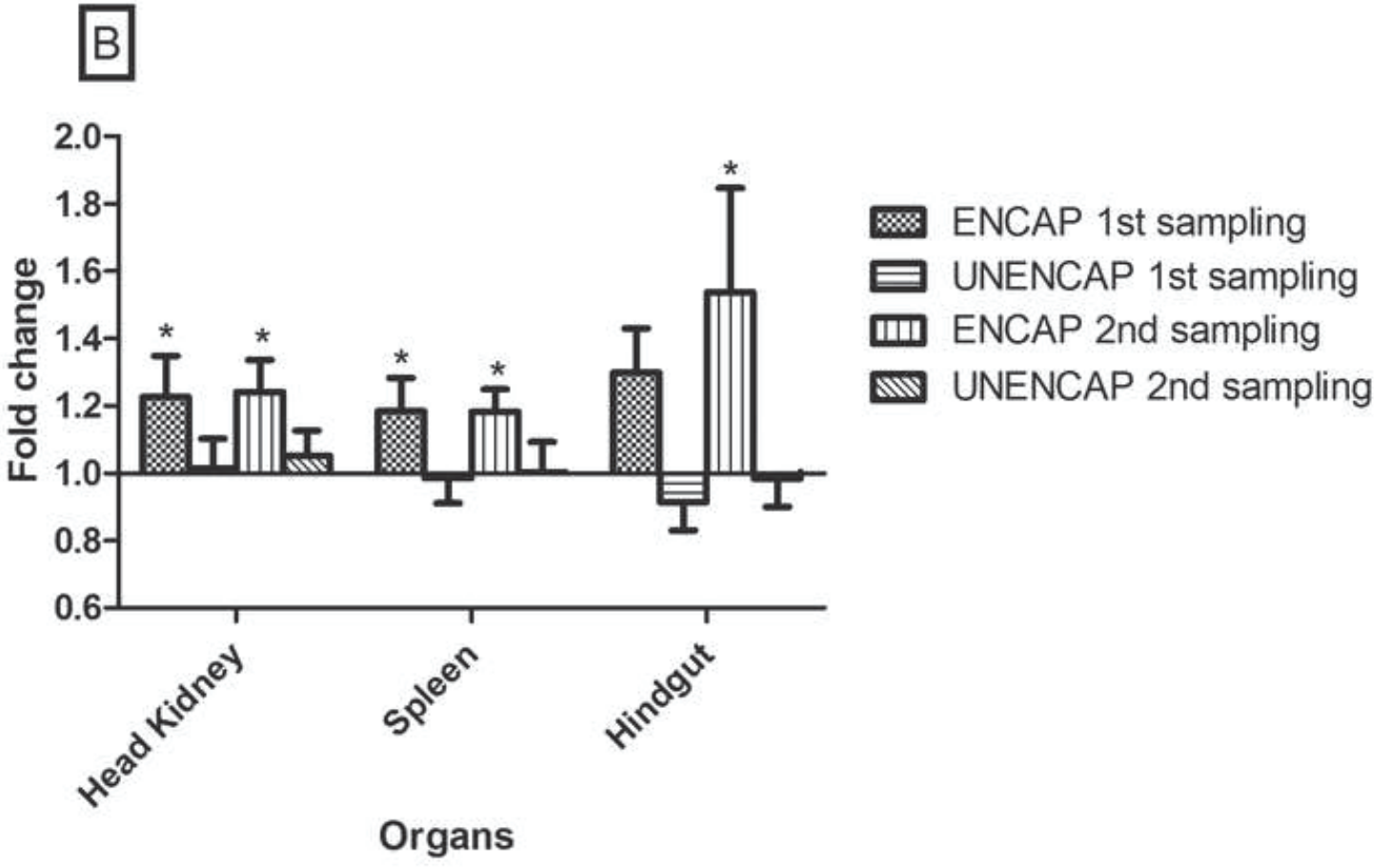

B
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
Fold change
*
*
*
*
*
Head Kidney
Spleen
Hindgut
Organs
ENCAP 1st sampling
UNENCAP 1st sampling
ENCAP 2nd sampling
UNENCAP 2nd sampling

Figure 3c

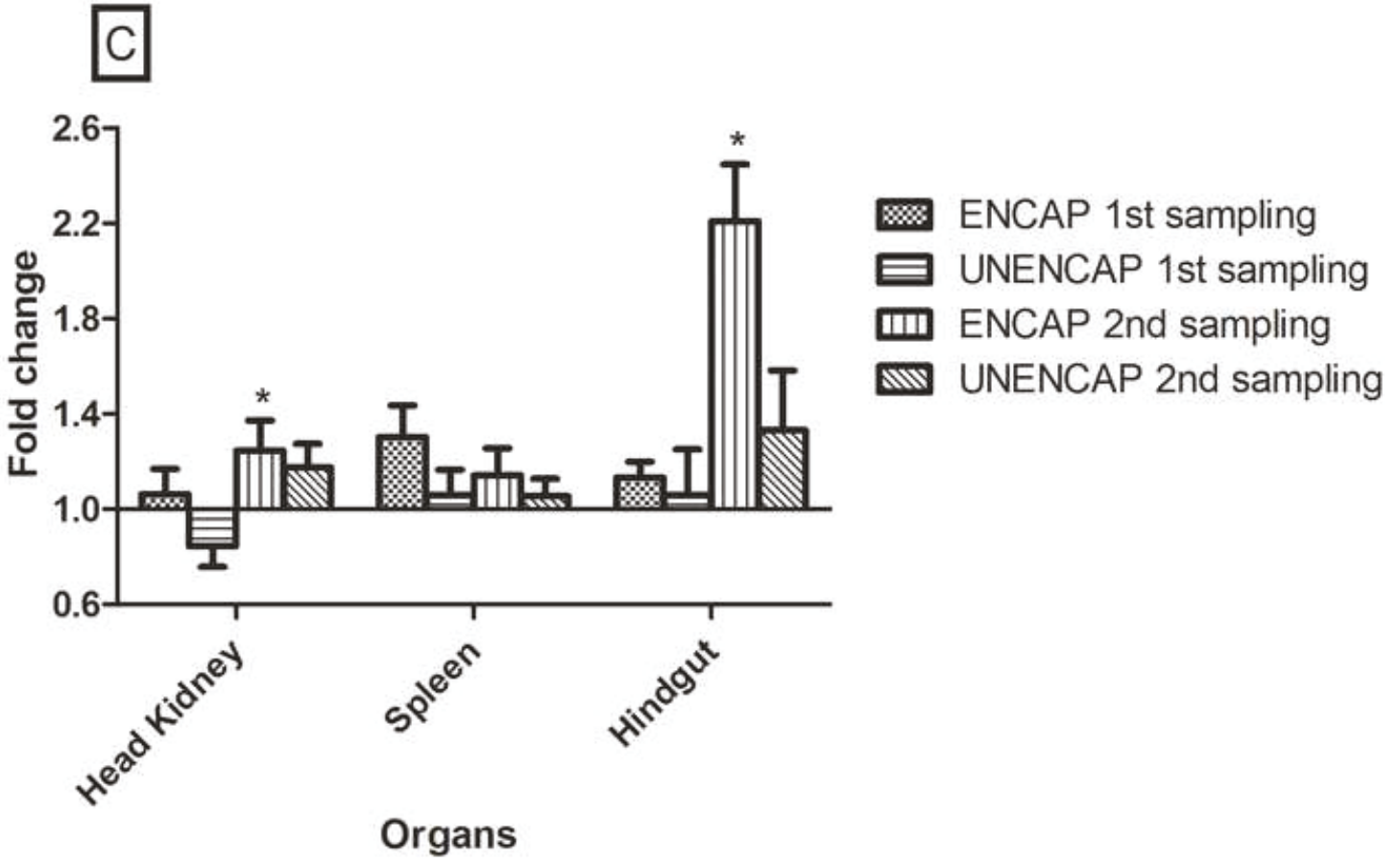

C
2.6
2.2
1.8
1.4
1.0
0.6
Fold change
*
*
Head Kidney
Spleen
Hindgut
Organs
ENCAP 1st sampling
UNENCAP 1st sampling
ENCAP 2nd sampling
UNENCAP 2nd sampling

Figure 3d

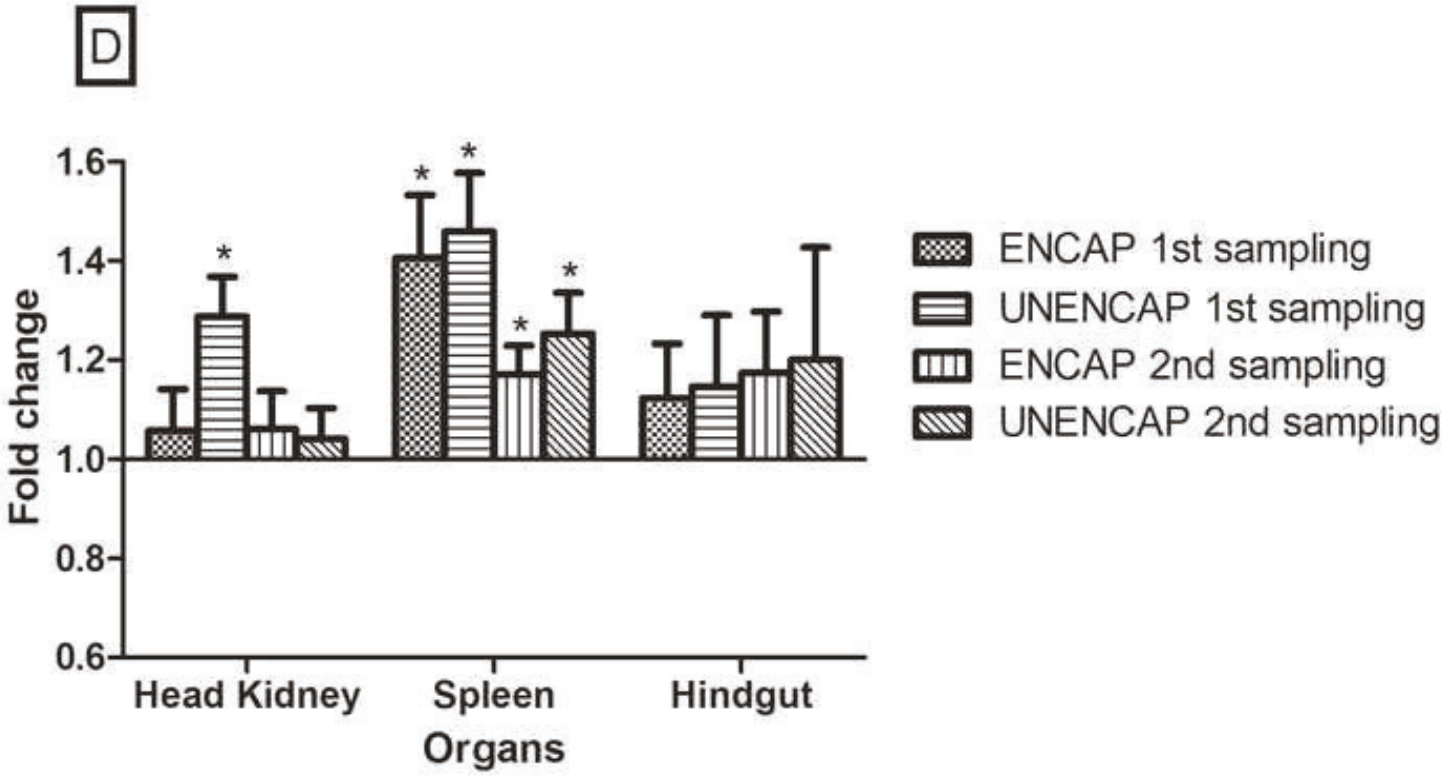

D
1.6
1.4
1.2
1.0
0.8
0.6
Fold change
Head Kidney
Spleen
Hindgut
Organs
ENCAP 1st sampling
UNENCAP 1st sampling
ENCAP 2nd sampling
UNENCAP 2nd sampling

Figure 4a

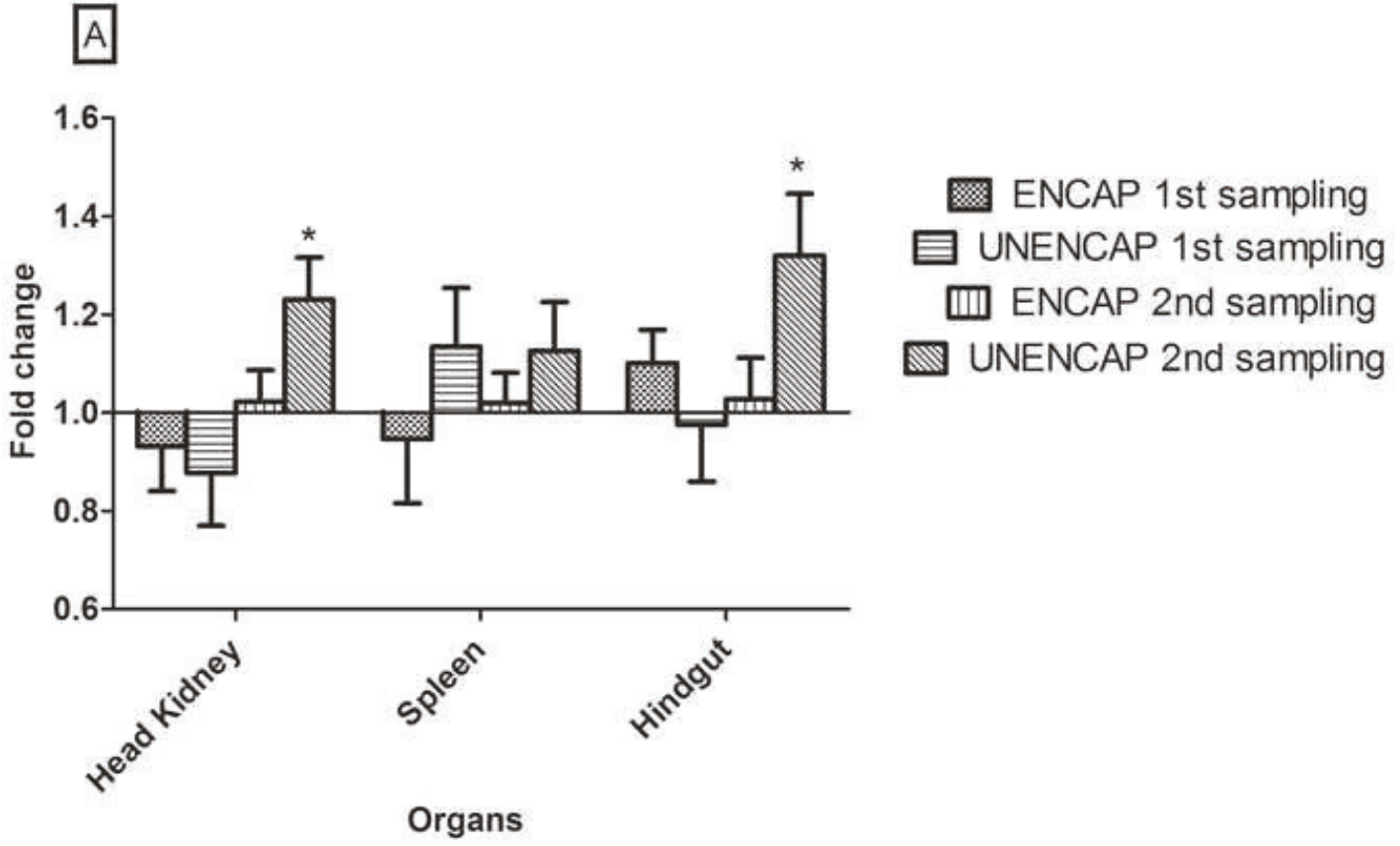

A
1.6
1.4
1.2
1.0
0.8
0.6
Fold change
*
*
Head Kidney
Spleen
Hindgut
Organs
ENCAP 1st sampling
UNENCAP 1st sampling
ENCAP 2nd sampling
UNENCAP 2nd sampling

Figure 4b

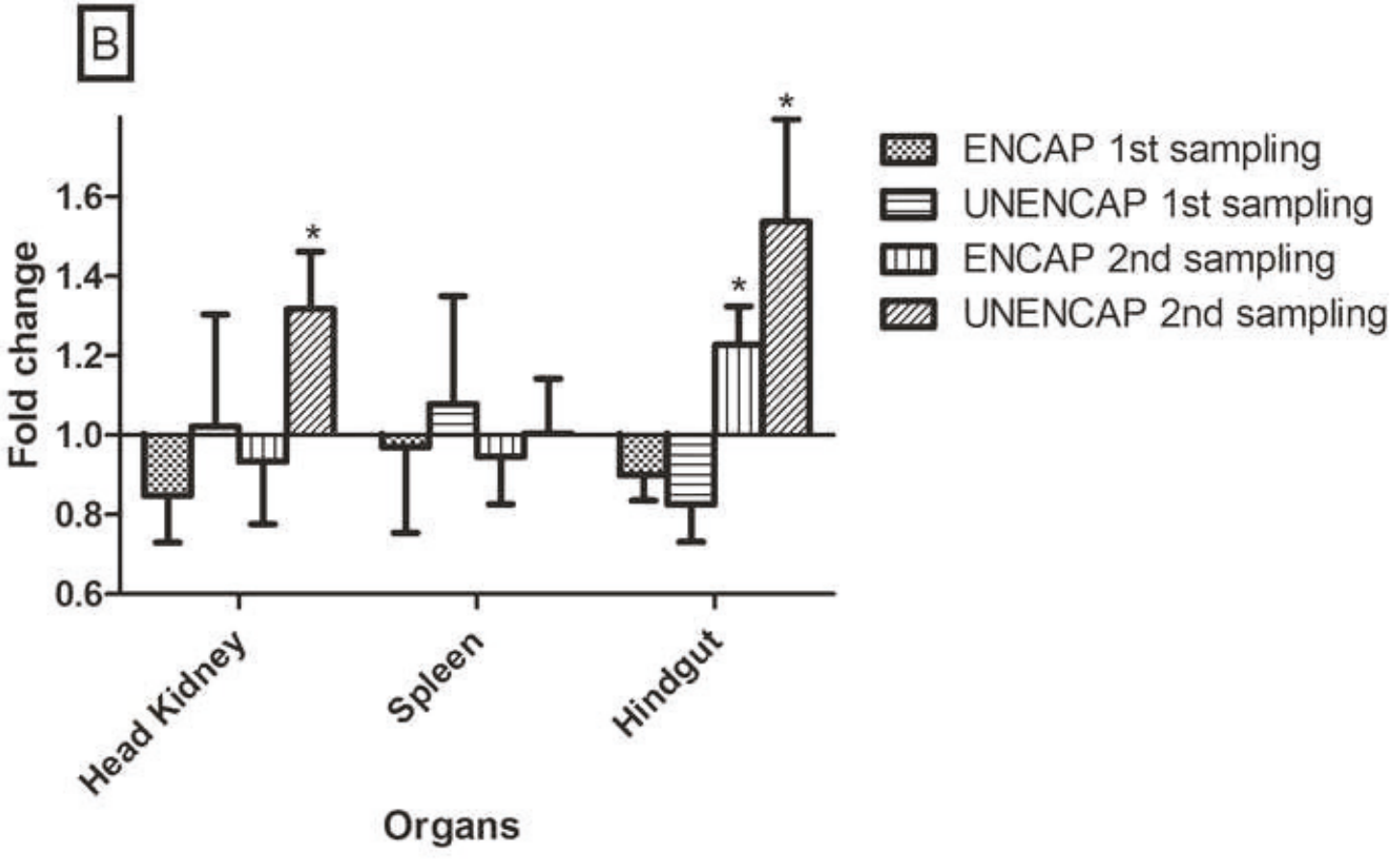

B
Fold change
1.6
1.4
1.2
1.0
0.8
0.6
*
*
*
Head Kidney
Spleen
Hindgut
Organs
ENCAP 1st sampling
UNENCAP 1st sampling
ENCAP 2nd sampling
UNENCAP 2nd sampling

Figure 4c

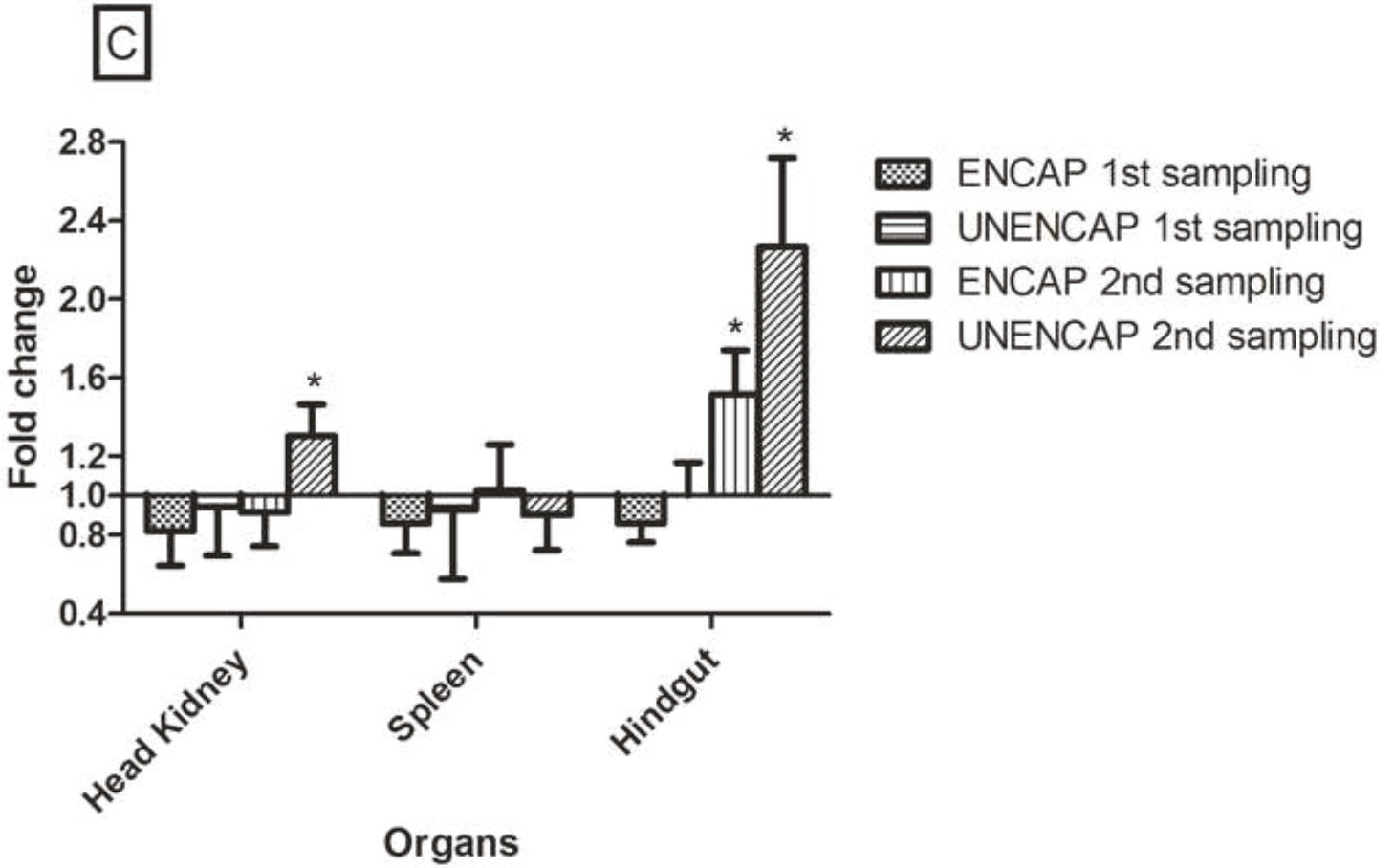
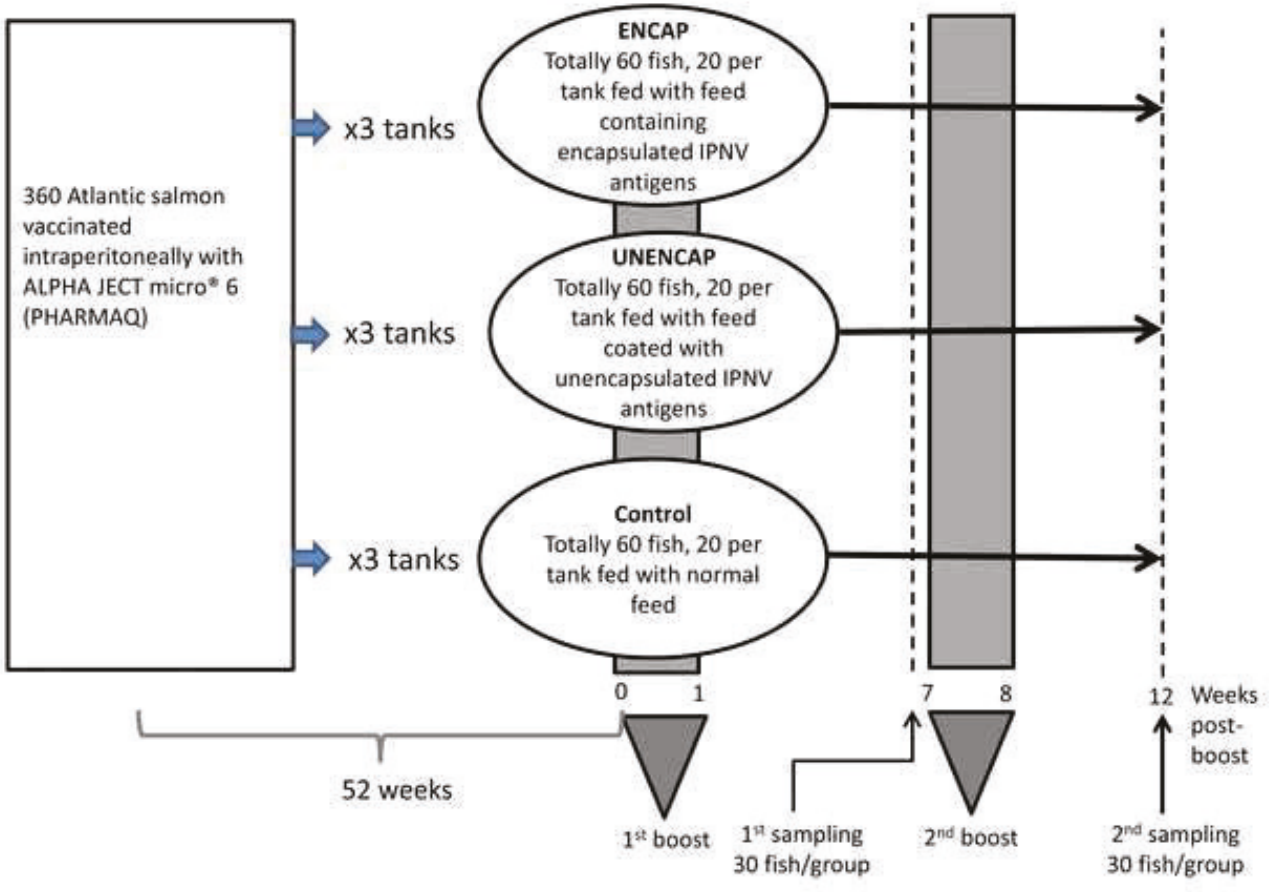

C
2.8
2.4
2.0
1.6
1.2
1.0
0.8
0.4
Fold change
*
*
*
Head Kidney
Spleen
Hindgut
Organs
ENCAP 1st sampling
UNENCAP 1st sampling
ENCAP 2nd sampling
UNENCAP 2nd sampling

Figure 5


360 Atlantic salmon vaccinated intraperitoneally with ALPHA JECT micro® 6 (PHARMAQ)
x3 tanks
x3 tanks
x3 tanks
ENCAP
Totally 60 fish, 20 per tank fed with feed containing encapsulated IPNV antigens
UNENCAP
Totally 60 fish, 20 per tank fed with feed coated with unencapsulated IPNV antigens
Control
Totally 60 fish, 20 per tank fed with normal feed
0
1
7
8
12
Weeks post-boost
52 weeks
1st boost
1st sampling 30 fish/group
2nd boost
2nd sampling 30 fish/group