UNIVERSITY OF TROMSØ UIT

NORWEGIAN COLLEGE OF FISHERIES SCIENCE

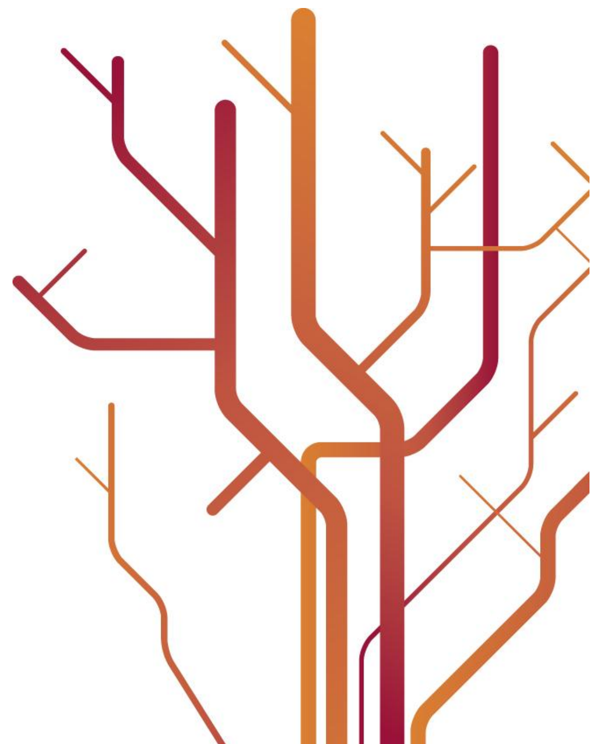
SAV Ag CpG/polyI:C formulated vaccination potentiate protective immune responses in Atlantic salmon

- **no additive or synergistic effect present when co-injecting a Rhabdovirus G-DNA vaccine**

Hanna Leena Thim

Master's Degree Thesis in Marine Biotechnology
(60 credits)

February 2013



Aknowledgements

Denna studien har utförts som en del av InNoVacc plattformen och har, under de senaste månaderna, utvecklats till en masteroppgave som i retrospektiv redan startade den 19 oktober 2011 med vaccineringen av 656 atlantiska laxar som därefter fick sätta livet till för att denna studie skulle bli en realitet.

Först ett stort tack till Jorunn som alltid tror på mig, pushar mig vidare och vänder på varenda liten och stor sten här på NFH för att få mig framåt – detta till trots för att jag är svensk! ☺ Du är en allvetandes, tuff och stark person, som har bättre koll på vad jag gjort och gör till trots för att du har X antal andra bollar i luften samtidigt. Det gör dig till en god, men smått ouppnåelig förebild!

Ett STORT tack till Stephané, Nina, Barbara och Glenn. Tack för all hjälp och underhållning i Bergen under detta projektet - och för en helt fantastisk middag på Bryggen! Och till Petter och alla andra på ”Intervet” - för ett härligt gäng Ni är!

Mehrdad, Dimi, Guro och Astrid. Tack för att ni alla är och har varit som levandes lexikon på labben vad jag än undrat över - ni gör mig till en bättre labbråtta för var dag som går!

Linn. Tack för alla fantastiska post-it notes som möter mig (till dygnets alla omöjliga timmar) här på kontoret om alla Ding’s och andra fantastiska Ting! ☺ Jag kommer sakna ditt sällskap!

Och så till Er jag inte nämner nu men som alltid är i mina tankar – Ni kommer förhoppningsvis få ert tack i min PhD :-S MEN, det finns två personer som aldrig kan nämnas för lite. Mamma och Pappa – vad hade jag varit utan er! Ni tror på mig, stöttar mig och står bakom mig i alla underliga ting jag tar mig för. Ni är världsbäst!

…fortsättning följer…

Abstract

Vaccines of today rely on adjuvant efficacy to provide a link between innate and adaptive immunity. There is a wide range of adjuvants with the potential to act synergistically. CpG oligodeoxynucleotides (ODN) and polyinosinic:polycytidylic acid (polyI:C) are both toll-like receptor (TLR) ligands signaling through a combination of pathogen recognition receptors (PRR) and in mammals CpG/polyI:C are known to induce T-helper 1 (Th1) and cytotoxic T-lymphocyte (CTL) responses. Currently, the commercial vaccine against salmonid alpha virus (SAV) is based on inactivated whole-virus antigen combined with oil (Montanide ISA), mainly stimulating humoral responses. CpG/polyI:C have already shown to be a potent adjuvant in an i.p. injected SAV antigen (Ag) vaccine. Here, the purpose was to differentiate between adjuvant and antigen induced protection for both water and oil formulated SAV Ag combined with CpG/polyI:C. Moreover, i.p. injected SAV Ag was also co-injected (i.m.) with Novirhabdovirus G DNA vaccine (vhsG), a potent inducer of innate antiviral responses in fish. This to observe if vhsG could enhance the immunity induced by the SAV Ag vaccine and also combined with SAV Ag CpG/polyI:C, to test whether vhsG could maximize the protection further. Expression of early antiviral genes, protection and elicited humoral responses were used to differentiate between the various vaccine formulations, pre- and post-cohabitant SAV challenge. SAV Ag alone and SAV Ag CpG/polyI:C provided full protection against SAV masking the objective to differentiate between Ag and adjuvant induced protection. Nonetheless, CpG/polyI:C again induced high innate immune gene expression (IFNa1, Vig-1, Mx and IFNγ) post vaccination and a potent induction of heat stable (neutralizing antibodies) and heat volatile humoral responses (complement) pre- and post-challenge. A clear reduction in heat stable humoral responses, parallel to heat volatile responses were seen for all treatments pre- and post-challenge, suggesting an importance of complement activation for SAV protection. The stimulatory effect of vhsG co-injected treatments on early innate immune gene expression was modest. Co-injection of vhsG with SAV Ag CpG/polyI:C did not provide any beneficial adjuvant effects, rather indicating an inhibitory interplay between CpG/polyI:C and vhsG. SAV Ag co-injected with vhsG did show a slight increase in humoral responses compared to SAV Ag alone. Results presented here, again demonstrate the immunostimulatory potency of CpG/polyI:C to be used as an adjuvant in viral vaccines for fish.

Abbreviations *(in order of appearance)*

SPDV *salmon pancreas disease virus*
PD *pancreas disease*
ORF *open reading frame*
SAV *salmonid alphavirus*
NAb *neutralizing antibody*
PRR *pattern recognizing receptor*
TLR *toll-like receptor*
Ag *antigen*
DC *dendritic cell*
MHC *major histocompatibility complex*
APC *antigen presenting cell*
ODN *oligodeoxynucleotide*
IFN *interferon*
HK *head kidney*
polyI:C *polyinosinic:polycytidylic acid*
ds *double stranded*
RIG-1 *retinoic acid-inducible gene 1*
MDA5 *melanoma differentiation associated protein 5*
Ab *antibody*
IHNV *infectious hemapoietic necrosis virus*
VHSV *viral hemorrhagic septicemia virus*
vhsG *VHSV glycoprotein (G) DNA vaccine*
i.p. *intraperitoneal*
i.m. *intramuscular*
PBS *phosphate buffered saline*
$TCID_{50}$ *tissue culture infective dose*
GMO *gene modified organism*
wpv *week post vaccination*
RT-qPCR *reverse transcriptase-quantitative polymerase chain reaction*
wpc *week post challenge*
Cq *threshold cycle*
OD *optical density*
CHSE-214 *Chinook salmon embryo cells*
HI *heat inactivated*
NHI *not heat inactivated*
MM *maintenance media*
MEM *minimum essential media*
FBS *fetal bovine sera*
ELISA *enzyme-linked immunosorbent assay*
RT *room temperature*
MAb *monoclonal antibody*
HRP *horse radish peroxidase*
OPD *peroxidase substrate*
RPPsc. *relative percent protection score*
NT *neutralizing titer*
TI *T-cell independent*
ADCMC *antibody dependent complement mediated cytotoxicity*
ASC *Ab-secreting cell*
SLPC *short-lived plasma cell*
LLPC *long-lived plasma cell*
ISG *IFN stimulated gene*

Table of contents

1 Introduction 1

2 Materials & Methods 4

2.1 Reagents and constructs 4

2.2 Fish 4

2.3 Vaccination and co-habitant challenge 4

2.4 SAV nsP1 qRT-PCR detection 6

2.5 Histopathology 6

2.6 RT-qPCR of immunegene expression 6

2.6.1 RNA isolation and reverse transcription 6

2.6.2 RT-qPCR 7

2.7 Viral neutralizing assay 8

2.8 Statistical analyses 9

3 Results 9

3.1 Protection 9

3.2 Vaccine-induced anti-SAV neutralizing humoral responses following immunization and SAV cohabitant challenge 13

3.3 Immune genes 14

4 Discussion 18

4.1 CpG/polyI:C maximizes both anti-viral immune gene expression post vaccination and protective humoral responses post cohabitant SAV challenge 18

4.2 Co-injection of vhsG with SAV Ag alone provided a slight increase in neutralizing responses while the opposite was evident for vhsG co-injection with SAV Ag CpG/polyI:C 20

4.3 vhsG treatments showed a weak induction of IFN stimulated gene expression from 12 to 48 hpv in spleen 21

4.4 vhsG does not contribute with any significant long term protective effects against SAV cohabitant challenge 22

5 Conclusion 23

References 24

Supplementary data 31

1 Introduction

Salmon pancreas disease virus (SPDV) is the causative agent for Pancreas disease (PD) which causes big economic losses throughout Norwegian, Scottish and Irish aquaculture industry. PD is a disease that affects both rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) and most often leads to PD outbreaks in post-smolts during their first year after sea transfer [1]. SPDV is an enveloped, single-strand positive sense RNA virus with an 11-12kb genome consisting of two large non-overlapping open reading frames (ORF) coding for non-structural and structural proteins [2]. Its taxonomic placement is in the *Alphavirus* genus within the *Togaviridae* family [3] and due to its placement it is largely referred to as salmonid alpha virus (SAV). Sequencing and phylogenetic studies has up till today shown that there are 6 subtypes of SAV (SAV1 to 6) [4]. SAV3 has been exclusively found in Norway and in 2011 it was made clear that a SAV2 like subtype also caused outbreaks in Atlantic salmon after sea water transfer on various locations in the mid-west region of Norway [5, 6]. The epizootic of SAV subtype 1, 2, 4, 5 and 6 is well described in the literature [4, 5]. Clinical signs of PD in Atlantic salmon are often lethargy and anorexia [7], mortality rates during outbreaks vary greatly [8, 9] and the severity depends on several factors, as for example fish strain [7]. Histopathological signs of PD are characterized by lesions in pancreatic acinar tissue, heart and later also in skeletal muscle [7, 10]. Several studies has demonstrated protective immune responses against SAV in salmonids, both experimentally and in the field [11, 12] and the protection has shown to be associated with antibody mediated immunity and neutralizing antibodies (NAb) [11, 13, 14]. Passive immunisation of SAV in live fish has provided evidence of protective NAbs [15] and since then various vaccination strategies against SAV have been tested both on Atlantic salmon and rainbow trout, such as attenuated live vaccines [16], formalin-inactivated viral vaccines [17] and also recombinant vaccines [18]. An inactivated whole-virus vaccine based on an Irish SAV1 isolate has been on the Norwegian, Irish and British market since 2002 [17, 19].

The mechanisms by which vaccines induce persistent immune responses has for a long time been unknown. However, through the discovery of pattern recognition receptors (PRRs), including the toll-like receptor (TLR) family, it is now clear that activation of TLRs present on dendritic cells (DC) induces production of inflammatory cytokines, upregulation of major histocompatibility complex (MHC) molecules and co-stimulatory signals, providing the crucially important link between innate and adaptive immunity [20, 21]. The knowledge about TLRs has significantly contributed to advance the field of adjuvant research and today it is

clear that signaling through a combination of PRRs renders a synergistic effect and increase adjuvant efficacy [22, 23]. One successful example of that is the empirically developed vaccine against yellow fewer, YF-17D, which activate DCs through multiple TLRs to stimulate the production of proinflammatory cytokines [24]. Both mammalian and aquaculture vaccines used today rely on adjuvant efficacy [25-27] and in salmonid aquaculture oil-based adjuvants are most widely used [19, 28]. Their function are based on creating a depot of Ag, which improves Ag delivery to antigen-presenting cells (APC) or by attracting effector cells to the site of injection [25, 28]. However, side effects due to oil-adjuvants have been reported and are expressed both physiologically and morphologically [29-31] and it is therefore desired to develop efficient adjuvants without side effects.

Research in our lab has been focused on synthetic TLR-ligands, CpG oligodeoxynucleotides (ODNs), which bind to the TLR9. Teleost TLR9 has been described in many species, including Atlantic salmon [32]. CpG ODNs have in fish been shown to activate different immune processes including type I IFN along with antiviral activity [33, 34], macrophage activation [35], cytokine production, activation of other immune related genes [36, 37] and cell proliferation [38-40]. Illiev et al. [41] showed by microarray analysis that several genes important for APC functions are upregulated in CpG-stimulated salmon head kidney (HK) leukocytes, proposing that CpGs may be used to improve vaccines for farmed salmon. As previously mentioned, synergistic immune stimulating effects have been reported by several studies when combining two or more microbial products, which elicit stronger and more robust immune responses upon encounter with a pathogen [42, 43]. One other potent TLR-ligand is polyinosinic:polycytidylic acid (poly I:C), which imitates double stranded (ds) RNA and is recognized by TLR3 in endosomes of specialized cells or by other receptors (RIG-1 and MDA5) that are expressed in the cytosol of all somatic cells [44]. Poly I:C is an effective inducer of type I IFN [44] and studies on poly I:C alone in Atlantic salmon have shown that it can induce resistance against viral infections [34, 45]. In primary chicken monocytes it has been reported that when combining CpG and poly I:C a synergistic induction of both antiviral and antibacterial inflammatory immune responses was present [46]. In accordance with this, Strandskog's et al. [47] studies on Atlantic salmon have displayed that a combined treatment with CpG/poly I:C induces synergistic upregulation *in vivo* of the immune genes IFNa, Mx, CXCL10, IL-1β, IFNγ and CD83. Moreover, CpG/polyI:C significantly enhanced protection on its own [48] and when formulated in a SAV whole-virus Ag formulation [49] the combo significantly increased antibody mediated

clearance of SAV in blood, with the effect that no SAV specific heart lesions were developed during the course of the challenge. This strongly indicates that a humoral antibody (Ab) response is important for protection against SAV and that this protection can be boosted by CpG/polyI:C. Intriguingly, we also found indications of cell-mediated immune responses activated by CpG/polyI:C. The T cell associated gene IFNγ was upregulated in head kidney and spleen and although no neutralizing responses were present after challenge for the CpG/polyI:C alone treatment, significant protection visualized by a reduced amount of SAV induced heart lesions were provided. Since the SAV Ag formulation alone was fully protective in our previous study we could not discriminate between Ag-induced protection or adjuvant induced protection. Therefore, in this present study a lower Ag dose estimated to provide about 70% protection alone were used and hypothesised to be able to show a dose sparing effect between SAV Ag formulation alone compared to SAV Ag formulated with CpG/poly I:C.

The DNA vaccine based on the Novirhabdovirus glycoprotein (G) have well-documented effects and induce long-lasting protection against infectious hemapoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) [50]. Interestingly, this DNA vaccine effectively induce IFN related mechanisms, providing an early non-specific antiviral protection [50-52] and has been shown to induce an antiviral state in fish [53] that efficiently prevents infection from other types of virus [54]. Therefore, we also wanted to compare adjuvant and immunostimulatory effects between an i.p. injection of SAV Ag with or without CpG/polyI:C in parallel to an i.m. injection of the VHSV G (vhsG) protein encoded as a DNA vaccine given at the same time. This to determine if the effects of vhsG and CpG/polyI:C could be additive or synergistic. Moreover, oil-adjuvanted SAV Ag formulations were included to determine if i.m. co-injection of vhsG protein could contribute to increase the specific protection against SAV compared to SAV Ag Oil. This due to reported oil-depot effects with SAV Ag Oil formulations [49]. This trial was performed as a cohabitant SAV challenge in Atlantic salmon, which best mimics a natural infection. In addition, as opposed to i.p. challenge, a waterborne cohabitation challenge avoids the assumed bias in measured protection due to a possible interference of local nonspecific immunity (induced by oil adjuvant) that may occur when both the vaccine and challenge material is injected in the peritoneum.

2 Materials & Methods

2.1 Reagents and constructs

DNA plasmid pcDNA3-vhsG was kindly provided by Dr. Niels Lorenzen and diluted in 1x PBS to a concentration of 0.2mg/ml. The synthetic ds RNA; poly I:C (Merck, UK) and phosphorithioate-modified CpG-B oligonucleotide (2006T: T***C*****G***T***C*****G***T*T*T*T*G*T***C*****G***T*T*G*T***C*****G***T*T, Thermo Scientific, Ulm. Germany) were dissolved in TE buffer (10mM Tris, 1nM EDTA, pH 8) at 5 mg/ml and further diluted 10-fold in the final vaccine formulations. The SAV antigen formulations was provided by MSD Animal Health (Bergen, Norway) and prepared by propagating the SPDV type specific strain F93-125 (SAV1) in cell culture. $TCID_{50}$ was determined prior to virus inactivation. Montanide ISA 763 (Seppic, France) was used for the water-in-oil formulations.

2.2 Fish

The experimental challenge study was performed at ILAB's challenge lab facility at the University of Bergen (Høyteknologisenteret, Bergen, Norway), which fulfill the confinement conditions required for working with GMOs and DNA vaccines. Atlantic salmon, presmolt (Fister) with a mean weight of approximately 25-40 g at time of vaccination were kept in tanks supplied with running fresh water at 10-14ºC and fed with commercial dry feed (EWOS Bergen, Norway) based on appetite. The fish were starved for minimum 48 hours and anaesthetized with Metacainum (0.1 mg/ml bath treatment) prior to all handling.

2.3 Vaccination and co-habitant challenge

Fish were divided into 7 treatment groups (n = 64, 68, or 74 depending on required sampling size) and a saline injected control group (n = 78). As described in detail in Table 1, three treatment groups were i.p. injected with 100 µl of the SAV1 whole-inactivated virus Ag formulation, formulated with or without oil and/or 50 µg CpG/polyI:C. Moreover, three treatment groups were, in parallel to the i.p. injections described above, injected i.m. with 10 µg PcDNA3-vhsG plasmid diluted in 50 µl PBS (1x). One group received PcDNA3-vhsG plasmid alone and the control group were injected with 100 µl 0.9% saline. Fish were marked by fin and/or maxilla clipping and there were no mortalities observed after injection. A total of 656 fish were used and divided in 3 tanks; 1 tank for challenge (508 fish including shedders) and 2 tanks for early immune gene samplings (148 fish). At 6 weeks post

vaccination (wpv) 86 Atlantic salmon were injected i.p. with 0.2 ml SAV subtype 3, each receiving a viral dose of $1x10^3$ $TCID_{50}$ and added to the vaccinated fish as virus shedders (n=86 corresponds to 1/5 of the final population including shedders), see sampling overview in Figure 1. The shedders were marked with a red VIE label under the anal fin one week prior to challenge.

Table 1

Treatments, dose regime, number of fish and schedule for sampling of organs and blood

Treatment	Total # of fish	Analysis (# of fish)					
		Immune gene expression	nsP1 RT-qPCR	Histopathology	Neutralization		
		12 and 48hpv	3wpc[1]	5 and 6wpc[2]	6wpv	3wpc[1]	6wpc[2]
SAV Ag	74	8+8	10	15+15	13	15	15
SAV Ag Oil	64	8+8	10	15+15	8	10	15
SAV Ag CpG (50µg)/poly I:C (50µg)	74	8+8	10	15+15	13	15	15
SAV Ag PcDNA3-vhsG (10µg)	68	10+10	10	15+15	8	10	15
SAV Ag Oil PcDNA3-vhsG (10µg)	68	10+10	10	15+15	8	10	15
SAV Ag CpG (50µg)/poly I:C (50µg) PcDNA3-vhsG (10µg)	68	10+10	10	15+15	8	10	15
PcDNA3-vhsG (10µg)	68	10+10	10	15+15	8	10	15
Saline 0.9%	78	10+10	10	15+15	13	15	15

Treatment underlined, PcDNA3-vhsG, was i.m. injected parallel to i.p. injection of SAV Ag treatments. hpv; hours post vaccination, wpc; weeks post challenge. [1] Same fish sampled for nsP1 RT-qPCR as for NAb assay [2] Same fish were sampled for heart histopathology and for NAb assay

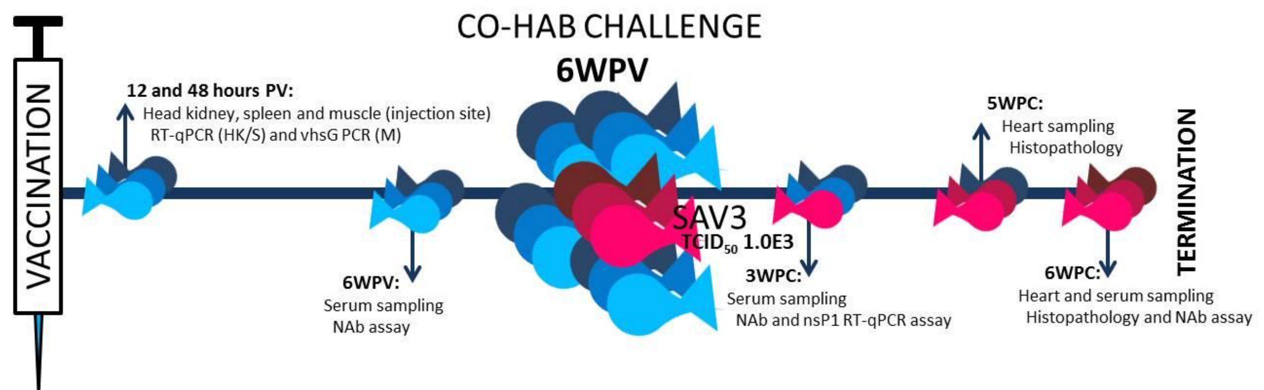


Figure 1. Timeline over the experimental set up and samplings from day zero to week 12. Detailed information about the vaccine formulations and number of sampled fish is presented in Table 1. The shedders (20% of the total amount of treated fish) were each injected with a $TCID_{50}$ of $1x10^3$ of SPDV SAV subtype 3. WPV; weeks post vaccination. WPC; weeks post challenge. HK; head kidney. S; spleen. M; muscle.

2.4 SAV nsP1 RT-qPCR detection

To measure SAV levels during the viraemic phase a quantitative RT-PCR (RT-qPCR) was performed on viral RNA extracted from sera 3 wpc from 10 individuals per group as described previously [48]. In short, 2 µl RNA extracted with QIAGENS QIAmp Viral RNA Kit was analyzed through a one-step RT-qPCR with SAV gene specific (Q_nsP1) TaqMan primers and probe [55]. RNA from SAV infected serum was included as a positive control and a sample was considered infected when the fluorescent signal increased above the threshold cycle (Cq) and below the cut off Cq-value of ≤37.5, based on the assay described by Hodneland and Endresen [55]. The threshold value was set to 0.2. Individual Cq-values were transformed to relative numbers by the following formula, where y represents the lowest Cq-value detected (i.e. the highest number of nsP1 transcripts) and where x is any of the other Cq-values detected:

$$RelCq(x) = 2^{(y-x)}$$

A sample were considered infected when it had a relative value between 1.0E00 and the cut off value of 3.0E-07 (x=37.5).

2.5 Histopathology

Samples for detecting SAV induced heart lesions were collected from 15 fish/group at 5 and 6 weeks post-challenge and immediately fixed in 3.5% formaldehyde in buffered saline at pH 7.0 (4.0g $NaH_2PO_4.{}_2H2_O$, 6.5g $Na_2HPO_4.{}_2H_2O$, 100ml 35% formaldehyde and 900ml distilled water). To evaluate the severity of SAV induced heart lesions a previously defined scoring system was used (no lesion: 0, minimal: 1, mild: 2, moderate: 3, severe: 4), where scores of 2 and more are defined to be specifically induced by a SAV infection (Christie et al, 2007). The scoring was done as a blinded experiment by Marian McLoughlin, Aquatic Veterinary Services, Belfast, Ireland.

2.6 RT-qPCR of immunegene expression

2.6.1 RNA isolation and reverse transcription

Spleen and HK were harvested from 8 fish per group at 12 and 48 hours post immunization and were stored on RNA later (Ambion, Applied Biosystems, USA) according to manufacturer's guidelines. Equally sized tissue samples were placed on 1ml Isol-RNA lysis

reagent (Prime 5, Hamburg, Germany) and homogenized (Precelleys 24, Bertin Technologies, France). The homogenate was mixed with 200µl chloroform and spun for 15 minutes at 12000g and 4°C before 350-450µl of the upper phase was placed on a column and the RNA was isolated according to the manufacturer's guidelines (NucleoSpin RNA II Kit; Macherey-Nagel, Germany). The RNA yield and purity was determined using Nanodrop ND-1000 Spectrophotometer (Nanodrop Tec., Wilmington, DA, USA). All samples had an $OD_{260/280}$ between 1.9 and 2.1. Four hundred nanogram total RNA was reverse transcribed (TaqMan Reverse Transcription Reagents kit; Applied Biosystems, USA) into cDNA using random hexamers primers in 30µl reaction volumes following the manufacturers guidelines.

2.6.2 RT-qPCR

Sequences of primers and probes and efficiencies of the assays used in this study are presented in Table 2. cDNA samples (2.5µl) were analysed in duplicates (target genes) or triplicates (endogenous control) in 20µl reactions on a 7500 Fast Real-Time PCR system. The Cq-threshold was automatically set to 0.2 for analysis of both endogenous and target genes. Relative expression and statistics were calculated using REST 2009 [56] which is based on Pfaffl's mathematical model [57]. Where Cq-values were compared between saline-injected fish and vaccine injected fish, correlated to the endogenous control EF1αβ and PCR-efficiency.

Table 2.

Primers and probe sequences for quantitative reverse-transcriptase PCR, PCR efficiency and GenBank accession number.

GENES	ASSAY	PRIMERS /PROBE	SEQUENCE (5'-3')	PCR EFF.	ACCESSION #
EF1aB	Fw/Rev 900µM	Forward*	TGCCCCTCCAGGATGTCTAC		
	Probe 250µM	Reverse*	CACGGCCCACAGGTACTG	2.0/2.0	BG933853
	SYBR Green*	Probe	[6FAM]AAATCGGCGGTATTGG[BHQ1]		
IFNa1	SYBR Green	Forward	CCTTTCCCTGCTGGACCA	2.0	AY2169594
		Reverse	TGTCTGTAAAGGGATGTTGGGAAAA		AY2169595
IFNγ	Fw/Rev 900µM	Forward	AAGGGCTGTGATGTGTTTCTG		AY795563
	Probe 250µM	Reverse	TGTACTGAGCGGCATTACTCC	2.0	
		Probe	[6FAM]TTGATGGGCTGGATGACTTTAGGA[BHQ1]		
Mx1/2	Fw/Rev 900µM	Forward	GATGCTGCACCTCAAGTCCTATTA		U66475
	Probe 250µM	Reverse	CGGATCACCATGGGAATCTGA	1.96	U66476
		Probe	[6FAM]CAGGATATCCAGTCAACGTT[BHQ1]		
Vig-1	Fw/Rev 900µM	Forward	AGCAATGGCAGCATGATCAG		
	Probe 250µM	Reverse	TGGTTGGTGTCCTCGTCAAAG	1.94	
		Probe	[6FAM]AGTGGTTCCAAACGTATGGCGAATACCTG[BHQ1]		
Q_nsP1	Fw/Rev 900µM	Forward	CCGGCCCTGAACCAGTT		
	Probe 250µM	Reverse	GTAGCCAAGTGGGAGAAAGCT	-	AY604235
		Probe	[6FAM]CTGGCCACCACTTCGA[BHQ1]		

2.7 Viral neutralizing assay

Serum samples from 8 to 15 individuals per group and time point (see Table 1 for details) were collected at 6 wpv, 3 and 6 wpc and examined for neutralizing responses. To do so, virus initially incubated with diluted sera was left to adhere to CHSE cells and after 8 days presence of cell-associated virus were detected. Individual sera from each group were pooled and half of the pooled sera were heat inactivated (HI). Assays for both HI and not heat inactivated (NHI) sera were repeated 3 times for all samples. In detail the assay setup was as follows: a 1:10 dilution of either HI or NHI serum (1:80 for CpG/polyI:C adjuvanted groups) was added in duplicate to wells on a 96-well microtiter plate and further diluted 2-fold with maintenance media (MM; MEM supplemented with 2% FBS) to the final dilution 1:320 (or 1:2560). To the wells containing 100µl of salmon serum dilutions, 100µl of virus supernatant SPDV (SAV subtype 1) was added leading to a dilution range from 1:20 to 1:640, or 1:160 to 1:5120 for the CpG/polyI:C treated groups. The virus supernatant had a concentration of approximately 6000 $TCID_{50}$ mL^{-1} for SAV. Virus positive controls, with the same virus concentration were incubated with MM, while cells incubated on MM alone were used as background controls. Plates were incubated for 2h at 15ºC with 5% CO_2. Thereafter, 25µl of the respective serum-virus mixtures were added to CHSE-214 cells grown in 96-well plates with 250µl MM. After 2 hours the incubation mix was removed and replaced with 100µl MM. Cell cultures were incubated at 15ºC with 5% CO_2 for 8 days before the level of viral multiplication was examined by ELISA. After removing the media, the cultures were fixed in 50/50 EtOH (96%)/acetone mix for 30 minutes at -20ºC. Fixed plates were washed 3 times with PBS-Tween (0.05%) and incubated for one hour at RT with 100µl/well primary MAb anti-E2 17H23 [58] (1:3000 in PBS-Tween (0.05%) supplemented with 1% dry milk). Again the plates were washed 3 times in PBS-Tween and 100µl/well of HRP conjugated anti-mouse Ig (Bio-Rad) diluted 1:1500 in PBS-Tween with 1% dry milk was added and left at RT for 2 hours. After a final triple wash 100µl/well of OPD substrate solution (4 tablets/12ml dH_2O supplemented with 5µl H_2O_2, DAKO) was added and the plates were incubated in darkness at RT for 15 minutes. The color development reaction was stopped using 50µl/well of 1M H_2SO_4 (Merck) and immediately after read in a spectrophotometer at OD 492nm. Neutralizing effects in serum were expressed as the highest reciprocal titers showing >50% reduction of the positive control OD value using the following formula:

$$50\%\ reduction\ OD = \frac{(\sum virus\ ctrl\ OD - \sum background\ ctrl\ OD)}{2}$$

2.8 Statistical analyses

All analyses were done in GraphPad Prism 5.0 if nothing else is mentioned. Differences in protection (SAV nsP1 RT-qPCR and histology) were statistically evaluated by Kruskal-Wallis Rank sum test with $p<0.05$ as significance limit. It was followed by the Dunn's post hoc test at a 5% level of significance. The histology test parameter used for statistical analysis was the severity of heart lesions, scored on the ordinal scale (0-4). Statistical analysis of the SAV nsP1 RT-qPCR used the individual Cq values of each group as test parameters. A modified expression of the relative percent protection score (RPPsc.) [48] was used to evaluate the level of protection against SAV induced by the tested treatments, based on the results obtained with the experimental methods (SAV induced heart lesions histology or SAV specific RT-qPCR assay). The advantage of this modified RPPsc. method is that the actual differences in degrees of severity of disease between the affected animals of the treated and control groups are taken into consideration. To evaluate any significant difference regarding the immune responses performed by RT-qPCR, the Relative Expression Software Tool (REST 2009 v.2.0.13) [56] were used.

3 Results

3.1 Protection

Six weeks after vaccination all fish were cohabitant challenged with SAV subtype 3 and vaccine induced protection was measured at 3, 5 and 6 wpc. At 3 wpc, when the viraemic phase is ongoing [59], sera were sampled and RNA from these sera were isolated and SAV nsP1 transcript levels detected by RT-qPCR. Heart tissue was sampled at 5 and 6 wpc to evaluate the severity of SAV induced heart lesions by histological scoring. At 3 wpc 70% of the sera in the saline injected group were SAV positive thus representing a successful challenge (Fig. 2). At 5 wpc (Fig. 3A) 60% of the fish receiving saline had SAV induced heart lesions and 3 out of 9 of these positive fish had severe lesions, while one week later (Fig.3B), 80% of the saline injected fish had SAV specific lesions and 10 out of 12 individuals had severe heart lesions. Four of the six SAV Ag treatment groups were fully protected against SAV at 3 wpc, as shown by the nsP1 RT-qPCR with no detectable Cq values, namely the water-formulated treatments; SAV Ag, SAV Ag CpG/polyI:C, SAV Ag vhsG and SAV Ag CpG/polyI:C vhsG. For the two oil-formulated groups; SAV Ag Oil and SAV Ag Oil vhsG,

20 and 10% of the fish had nsP1 positive sera, leading to RPPsc. of 71.4 and 85.7%, respectively. Furthermore, based on prevalence of viremia determined by nsP1 RT-qPCR there was a significant difference between all water- and oil-formulated SAV Ag treatments compared to saline treated fish ($P<0.05$ or 0.01, see Table 3) except for SAV Ag Oil. Prevalence of nsP1 positive fish in the group treated with an i.m. injection of vhsG alone was significantly higher, than the prevalence in the other treatment groups ($P<0.05$ or 0.001) except the saline group. Protection in the group treated with vhsG alone was less than in the saline injected fish with a RPPsc. of -0.28% (9 out of 10 fish positive for SAV in serum).

At 5 and 6 wpc protection based on reduction of the severity of SAV induced heart lesions was comparable to the protection shown through viraemic prevalence at 3wpc for all treatments. All water based formulations and also the SAV Ag Oil formulation, gave full protection at 5wpc with no or minimal lesions and they were all statistically different from both saline and vhsG treated fish ($p<0.05$ or 0.01, see Table 4 and 5). RPPsc. values for the vhsG alone treated fish was 11.1% at 5wpc at when 5 fish showed mild to severe lesions compared to 6wpc where 12 of 15 fish had mild to severe lesions (RPPsc. -8.3%). Three fish receiving SAV Ag Oil vhsG had moderate to severe lesions (RPPsc. 66.7%) and there was no significant difference between this group and any of the other treatments at 5 wpc. At 6wpc lesions in the SAV Ag Oil vhsG group were reduced and only 1 out of 15 fish showed moderate lesions (RPPsc. 83.3%). Interestingly, at 6 wpc the group treated with SAV Ag Oil had a RPPsc. of 58.3% and 5 fish showed mild to severe lesions, compared to 5wpc when the RPPsc. was 100%, with only two fish showing minimal heart lesions (no significant difference between SAV Ag Oil at 5 and 6 wpc).

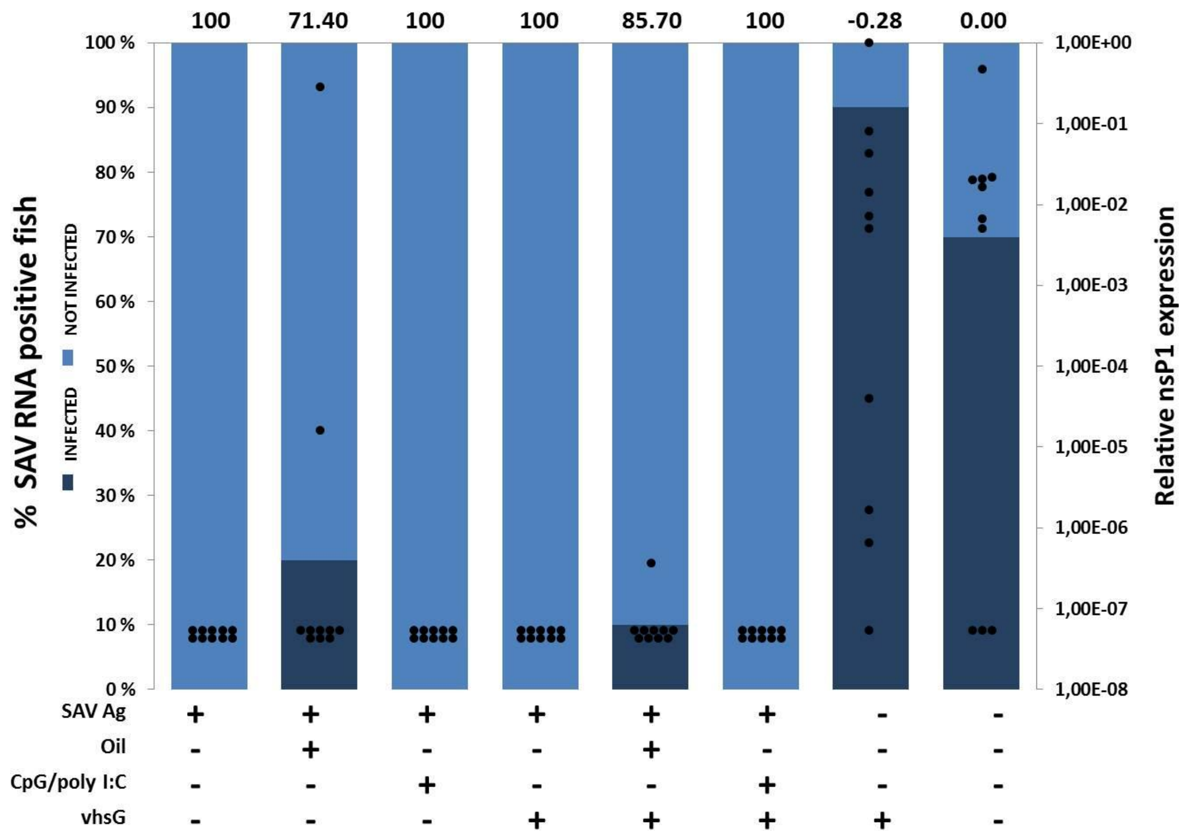


Figure 2. Protection against PD in vaccinated and control groups based on reduction of viraemic prevalence. Percentage of fish with SAV RNA positive sera (left y-axis) and relative SAV nsP1 expression (right y-axis) at 3wpc measured by SAV nsP1 RT-qPCR for each treatment group. Individual Cq-values were transformed to RelCq numbers (black dots) as described in Materials & Methods. One (1.0E00) indicates the highest presence of nsP1 transcripts. Sera below the dotted line (cut off; 3.0E-07) were considered negative and sera below the solid line had undetected Cq values. RPPsc. values are shown above the histogram corresponding to each group. + or - respectively indicates presence or absence of either SAV Ag, oil, CpG/poly I:C or vhsG.

Table 3.

Kruskal-Wallis and Dunn's post hoc test for SAV nsP1 RT-qPCR at 3wpc.

	SAV Ag Oil	SAV Ag CpG/polyI:C	SAV Ag vhsG	SAV Ag vhsG Oil	SAV Ag CpG/polyI:C vhsG	vhsG	Saline
SAV Ag	ns	ns	ns	ns	ns	***	**
SAV Ag Oil		ns	ns	ns	ns	*	Ns
SAV Ag CpG/polyI:C			ns	ns	ns	***	**
SAV Ag vhsG				ns	ns	***	**
SAV Ag vhsG Oil					ns	***	*
SAV Ag CpG/polyI:C vhsG						***	**
vhsG							Ns

ns: not significant *p < 0.05 **p < 0.01 ***p < 0.001

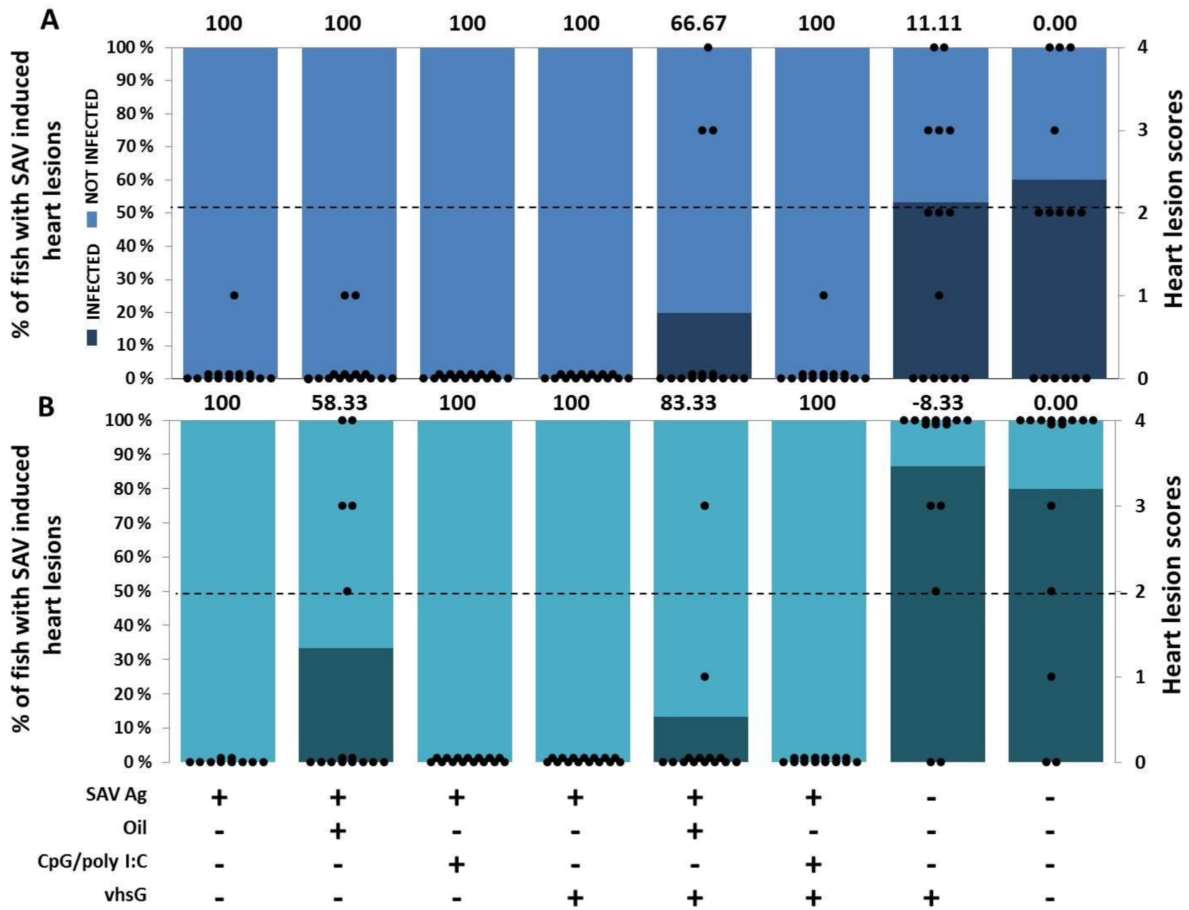


Figure 3. Protection against PD in vaccinated and control groups based on reduction in severity of SAV specific heart lesions. Percentage of fish with SAV induced heart lesions (left y-axis) and distribution of individual heart lesion scores (right y-axis) assessed by histology at 5wpc (A) and 6wpc (B) for each treatment group. A score of ≥2 was set as cut off (indicated by dotted line). Individual heart lesion scores are presented as black dots. RPPsc values are shown above the histogram corresponding to each group. + or - respectively indicates presence or absence of either SAV Ag, oil, CpG/poly I:C or vhsG.

Table 4.

Kruskal-Wallis and Dunn's post hoc test for heart histology at 5wpc.

	SAV Ag Oil	SAV Ag CpG/polyI:C	SAV Ag vhsG	SAV Ag vhsG Oil	SAV Ag CpG/polyI:C vhsG	vhsG	Saline
SAV Ag	ns	ns	ns	ns	ns	**	**
SAV Ag Oil		ns	ns	ns	ns	*	*
SAV Ag CpG/polyI:C			ns	ns	ns	**	**
SAV Ag vhsG				ns	ns	**	**
SAV Ag vhsG Oil					ns	ns	Ns
SAV Ag CpG/polyI:C vhsG						**	**
vhsG							Ns

ns: not significant *p < 0.05 **p < 0.01 ***p < 0.001

Table 5.

Kruskal-Wallis and Dunn's post hoc test for heart histology at 6wpc.

	SAV Ag Oil	SAV Ag CpG/polyI:C	SAV Ag vhsG	SAV Ag vhsG Oil	SAV Ag CpG/polyI:C vhsG	vhsG	Saline
SAV Ag	ns	ns	ns	ns	ns	***	***
SAV Ag Oil		ns	ns	ns	ns	*	*
SAV Ag CpG/polyI:C			ns	ns	ns	***	***
SAV Ag vhsG				ns	ns	***	***
SAV Ag vhsG Oil					ns	***	***
SAV Ag CpG/polyI:C vhsG						***	***
vhsG							ns

ns: not significant *p < 0.05 **p < 0.01 ***p < 0.001

3.2 Vaccine-induced anti-SAV neutralizing humoral responses following immunization and SAV cohabitant challenge

Presence of anti-SAV neutralizing responses in sera was measured at 6wpv and at 3 and 6 wpc by viral neutralization assays. Due to limited amount of sera pooled samples for each group were used in all tests and the assay was repeated three times, both for HI and NHI sera. Results presented are representative for all analyses. HI and NHI sera were analyzed to be able to differentiate between specific SAV neutralizing antibody responses or heat volatile factors (for example complement factors) mediating virus neutralizing responses. For the non-heat inactivated sera (Fig. 4A) detectable neutralizing titers (NT) were present from 6 wpv for all groups except SAV Ag Oil vhsG, vhsG alone and the saline treated fish. In agreement with earlier reports [49] the highest NT were detected in the SAV Ag CpG/polyI:C treated group with titers of 640 and 1280 at 6 wpv and 3 wpc, respectively, to 2560 at 6 wpc. SAV Ag CpG/polyI:C treated fish that also received the vhsG i.m. injection showed the second highest NAb titers of 640 and 320 at 6 wpv and 3 wpc, respectively and 640 at 6wpc. The vhsG and saline injected fish showed detectable NT after challenge, with titers of 80 and 160 at 3 and 6 wpc respectively for vhsG and 160 for saline treated fish at both time points. Furthermore, all vaccinated groups, except the SAV Ag vhsG Oil group, mounted a detectable neutralizing response before challenge. When inactivating the heat volatile factors the reduction in NT for heat stable factors showed the importance heat volatile factors provide for protection against SAV (Fig. 4B). SAV NAbs were not detected for SAV Ag, SAV Ag Oil and SAV Ag Oil vhsG treatments, while SAV Ag vhsG gave detectable NAb responses at all three samplings. Again, treatments with CpG/polyI:C provided a potent humoral response with NAb titers ranging from 640 at 6 wpv to 1280 at 6 wpc for SAV Ag CpG/polyI:C and for SAV Ag CpG/polyI:C vhsG the generation of NAbs was consistent with a titer of 160 for all sampling points. Fish treated with vhsG and saline, where 80-87% of the fish in both groups had

positive SAV specific heart lesions at 6 wpc (Fig. 3B), had detectable NAb titers at 6wpc (80 for both treatments), which represents the natural immune response against a SAV infection.

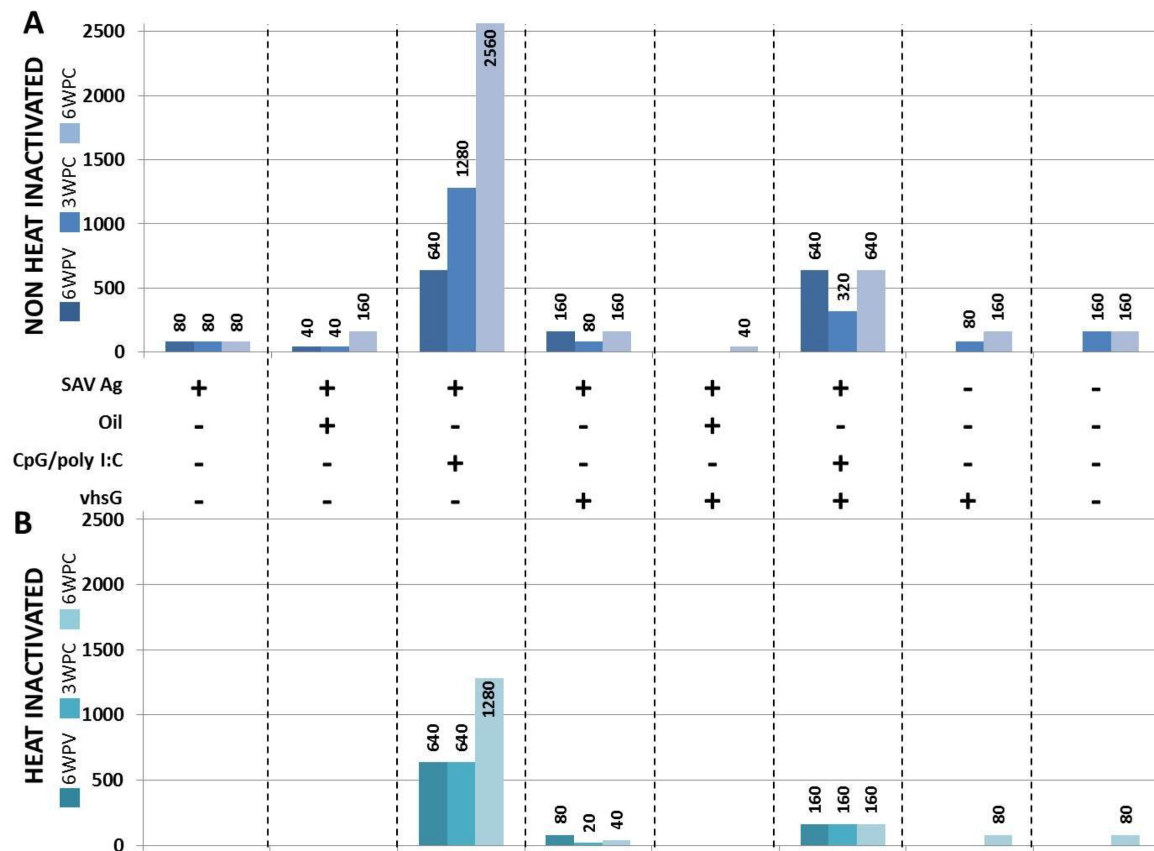


Figure 4. Vaccine induced anti-SAV neutralizing titers from non-heat inactivated (A) and heat inactivated (B) sera, collected at 6 wpv and 3 and 6 wpc. Titers representing 50% reduction, calculated as described in Materials & Methods, are shown above the histogram corresponding to each treatment. + or – respectively indicates presence or absence of either SAV Ag, Oil, CpG/polyI:C or vhsG.

3.3 Immune genes

Innate immune gene RT-qPCR analysis of head kidney (HK) and spleen samples harvested during this study (outline in Fig. 1 and Table 1) confirmed results obtained in previous studies [48, 49] and again, CpG/polyI:C adjuvanted treatments gave a strong induction of IFNγ in head kidney (HK; Fig. 5) and spleen (Fig. 6), which cannot be detected in any of the other groups. Moreover, upregulation of IFNa1 in the CpG/polyI:C treated groups was significantly higher at 12 hpv than 36 hours later in both HK (Fig. 5A; Table 6) and spleen (Fig. 6A; Table 7) and at the latter time point a 10-fold reduction was seen. This is consistent with the knowledge about type I IFN as an early induced antiviral actor and in

accordance the antiviral genes Vig-1 and Mx (IFN type I inducible proteins) were still highly expressed at 48 hpv in the CpG/polyI:C treated groups, indicating an upregulation upon IFNa1 stimulation. Generally, the immune gene expression pattern seen for SAV Ag alone and for the other four formulations without CpG/polyI:C, was at a moderate level in both organs. Previously, undetectable levels of IFNa1, IFNγ and Mx in HK and spleen of SAV Ag alone treated fish have been described at 5 days post vaccination [49]. Here in HK, SAV Ag alone induced a low, but significant upregulation of IFNγ, Vig-1 and Mx at 48 hpv compared to control fish (Fig. 5B). Immune gene responses in HK for vhsG immunized fish were in general as moderate as treatments without i.m. injection of vhsG, except when vhsG was co-injected with SAV Ag CpG/polyI:C. Compared to SAV Ag CpG/polyI:C the immune gene expression patterns for SAV Ag CpG/polyI:C vhsG were similar in both organs, except a slight but significant upregulation of IFNa1 and Vig-1 in HK and of IFNγ in spleen at 48 hpv. At 12 hpv IFNγ and Vig-1 expression were significantly lower for SAV Ag vhsG in HK compared to both saline and SAV Ag alone. Both type I and type II IFN were down regulated in HK at 48 hpv for the vhsG alone treatment compared to SAV Ag alone with no significant difference in the expression profile compared to saline, except for an upregulation of Vig-1. In spleen, at 48 hpv, IFNa1 was significantly up regulated in all vhsG treated groups compared to SAV Ag alone (Fig. 6B). Besides that, the trends in spleen were similar to those seen in HK.

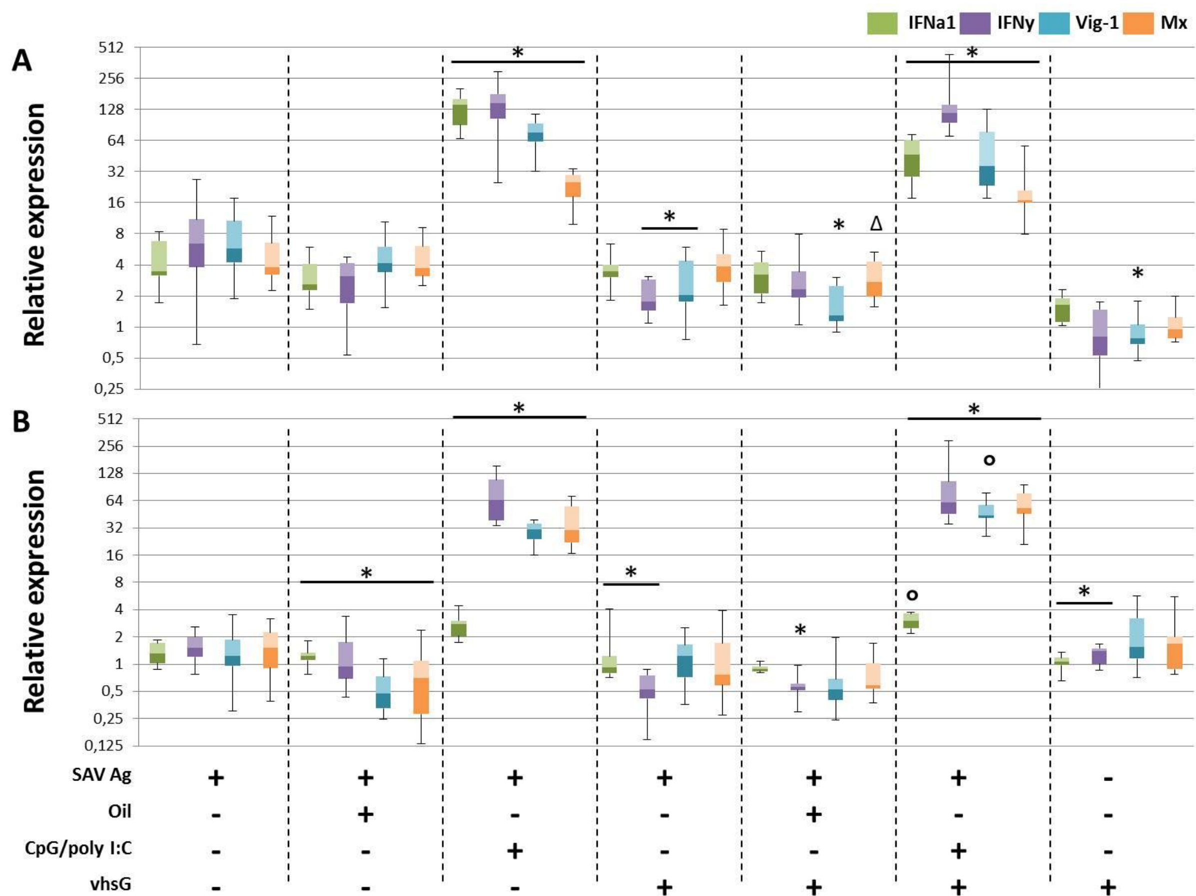


Figure 5. Relative expression of IFNa1, IFNγ, Vig-1 and Mx in head kidney at 12h (A) and 48h (B) for all treatments compared to saline treated fish and the expression is normalized to reference gene EF1aB. Relative expression is presented as whisker plots calculated from fold induction by Pfaffel's method (see Materials and Methods) and significant up or down regulation are based on data from REST2009. Significant difference for all treatments compared to SAV Ag is highlighted with an * and against SAV Ag vhsG as Δ and against SAV Ag CpG/polyI:C as °. + or - indicates presence or absence of either SAV Ag, oil, CpG/poly I:C or vhsG, respectively.

Table 6.

Significant up and down regulation (p-values obtained from REST2009) in HK at 12 and 48h for all genes and all formulations compared to the saline treatment.

HK 12h	vs.	SAV Ag	SAV Ag Oil	SAV Ag C/P	SAV Ag vhsG	SAV Ag vhsG Oil	SAV Ag C/P vhsG	vhsG
Saline	*IFNa1*	0,031↑	0,577	0,000↑	0,587	0,343	0,000↑	0,329
	IFNy	0,197	0,735	0,000↑	0,000↓	0,391	0,000↑	0,950
	Vig-1	0,057	0,047↑	0,000↑	0,000↓	0,000↓	0,000↑	0,769
	Mx	0,076	0,000↑	0,000↑	0,058	0,535	0,000↑	0,077

HK 48h	vs.	SAV Ag	SAV Ag Oil	SAV Ag C/P	SAV Ag vhsG	SAV Ag vhsG Oil	SAV Ag C/P vhsG	vhsG
Saline	*IFNa1*	0,000↓	0,289	0,000↑	0,809	0,000	0,000↑	0,238
	IFNy	0,000↑	0,011↑	0,000↑	0,250	0,320	0,000↑	0,622
	Vig-1	0,050↑	0,225	0,000↑	0,078	0,345	0,000↑	0,022↑
	Mx	0,046↑	0,889	0,000↑	0,102	0,051	0,000↑	0,139

Highlighted cells marked with ↑ indicate significant up regulation and with ↓ indicate significant down regulation ($p<0.05$)

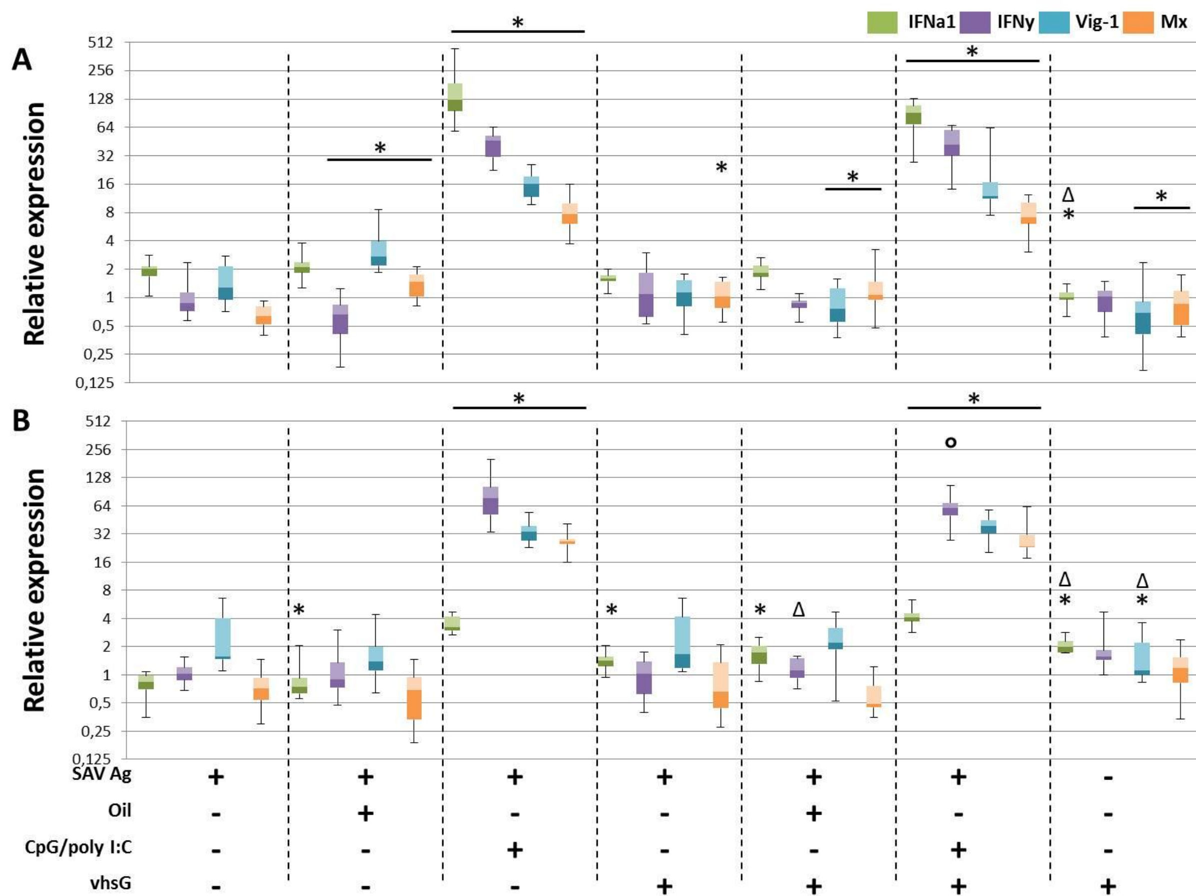


Figure 6. Relative expression of IFNa1, IFNγ Vig-1 and Mx in spleen at 12h (A) and 48h (B) for all treatments compared to saline treated fish and the expression is normalized to reference gene EF1aB. Relative expression is presented as whisker plots calculated from fold induction by Pfaffel's method (see Materials and Methods) and significant up or down regulation are based on data from REST2009. Significant difference for all treatments compared to SAV Ag is highlighted with an * and against SAV Ag vhsG as Δ and against SAV Ag CpG/polyI:C as °. + or - indicates presence or absence of either SAV Ag, oil, CpG/poly I:C or vhsG, respectively.

Table 7.

Significant up and down regulation (p-values obtained from REST2009) in spleen at 12 and 48h for all genes and all formulations compared to the saline treatment.

Sp 12h	vs.	SAV Ag	SAV Ag Oil	SAV Ag C/P	SAV Ag vhsG	SAV Ag vhsG Oil	SAV Ag C/P vhsG	vhsG
Saline	*IFNa1*	0,678	0,715	0,000↑	0,774	0,798	0,000↑	0,500
	IFNy	0,291	0,000↓	0,000↑	0,291	0,000↓	0,000↑	0,074
	Vig-1	0,007↑	0,000↑	0,000↑	0,865	0,243	0,000↑	0,055
	Mx	0,000↓	0,900	0,000↑	0,379	0,360	0,000↑	0,073

Sp 48h	vs.	SAV Ag	SAV Ag Oil	SAV Ag C/P	SAV Ag vhsG	SAV Ag vhsG Oil	SAV Ag C/P vhsG	vhsG
Saline	*IFNa1*	0,174	0,164	0,000↑	0,195	0,000↓	0,008↑	0,000↓
	IFNy	0,137	0,735	0,000↑	0,525	0,008↑	0,000↑	0,058
	Vig-1	0,000↑	0,011↑	0,000↑	0,001↑	0,000↑	0,000↑	0,167
	Mx	0,450	0,000↓	0,000↑	0,016	0,172	0,000↑	0,733

Highlighted cells marked with ↑ indicate significant up regulation and with ↓ indicate significant down regulation (p<0.05)

4 Discussion

4.1 CpG/polyI:C maximizes both anti-viral immune gene expression post vaccination and protective humoral responses post cohabitant SAV challenge

The primary goal of this study was to assess the potential of CpG/polyI:C as an adjuvant for a SAV vaccine based on inactivated whole-virus formulation. However, the full protection induced by the SAV Ag alone formulation obstructed this intention. Nonetheless, the potency of CpG/polyI:C to trigger both humoral and cellular immune responses was clearly seen during the course of the study. This through a distinct generation of neutralizing responses and a strong immune gene expression of both innate (IFNa1, vig-1 and Mx) and adaptive (IFNγ) genes at 12 and 48 hours post vaccination compared to the other treatments. Oil adjuvanted groups showed lower protection levels (RPP.sc. from 58.3-85.7%) and lower neutralizing titers (25-70% reduction) compared to the group receiving the equivalent water based formulation. The differences could be explained by a depot effect, i.e. slower release of Ag. The immunological mechanisms behind the full protection provided by SAV Ag alone has been discussed earlier [49] and in short, since SAV Ag alone did not induce an innate immune gene response it suggested that there was no significant induction of cellular adaptive immune responses. Therefore, it was reasoned that full protection had been provided through generation of T-cell independent (TI) NAb, considering the neutralizing response provided by SAV Ag alone. In this current study, a moderate induction of IFNa1, Mx, Vig-1 and IFNγ was evident in HK of SAV Ag alone treated fish at 12 hpv, which had declined at 48 hpv. Thereby, the innate immune response at 5 dpv, when Thim et al. [49] measured responses could have diminished already. The effect the moderate induction of innate responses has on cellular immunity later out in the challenge is complex to interpret, owing to the limited knowledge of cellular immunity against viral infections in fish. As an enveloped virus, SAV encodes several membrane bound glycoproteins, and by functioning as a whole-virus Ag vaccine multiple antigenic receptors on B-cells could have been cross-linked to activate a TI NAb production. That hypothesis is questioned here since NHI sera from SAV Ag treated fish displayed neutralizing activity, while there were no detectable neutralizing activity in SAV Ag alone HI sera. Interestingly, neutralizing responses in HI sera were reduced by half in all treatments when compared to NHI. Indicating that heat stable responses could still be present in SAV Ag alone treated fish, merely below the detection limit of the assay at hand. This difference between NHI and HI sera emphasizes the importance of a heat volatile factor involvement in clearance of virus in SAV infected fish, as previously suggested by Desvignes

et al [13]. In the article by Thim et al. [49] it was further discussed that CpG can function as a B-cell mitogen and potentiate Ab secretion by providing a second signal (through TLR9) to already activated Ab secreting B-cells. Here, heat stable NAb production (HI sera) contributed to approximately half of the total neutralizing response in the SAV Ag CpG/polyI:C adjuvanted group and the highest generation of heat stable NT compared to all other treatments. Which support the concept of CpG as a B-cell mitogen able to potentiate Ab secretion. Moreover, complement factors are heat labile factors known to inhibit Alphavirus infections [60]. There has been evidence of non-neutralizing Ab providing protection through Ab dependent complement mediated cytotoxicity (ADCMC) to for example Alphavirus [61, 62] and retroviruses [63]. Any ADCMC effect present here, could be detected by adding NHI sera from unchallenged Atlantic salmon to HI sera from all treated groups. If ADCMC is involved, the neutralizing capacity of the HI sera would be restored. In addition, Mangsbo et al. [64] has shown that CpG ODN 2006 (as used here) activates complement through elevated C3a and C5a levels in mice, indicating classical complement activation. In Atlantic salmon, induced levels of complement component C4 (classical pathway) has been generated in both saline and CpG/polyI:C treated fish after SAV challenge [48]. This further underlines what is presented here, that complement in general might aid in clearance of SAV. The potency of CpG/polyI:C is further highlighted by its ability to activate innate/adaptive immune gene responses. Including the supporting data provided here, of CpG as a B-cell mitogen, previously shown by Strandskog et al. [65], and since CpG/polyI:C appear to enhance the generation of heat volatile factors, i.e. complement, CpG/polyI:C display a wide range of protective mechanisms making it a prospective adjuvant combo. Humoral responses for SAV Ag CpG/polyI:C were stable or increased by double from pre challenge to 3 wpc and 6wpc. How do CpG/polyI:C affect the elevated generation of humoral responses post vaccination and the increase seen post challenge? This will be further studied in ongoing trials and it is intriguing to ponder upon the possible dynamic of B-cell populations in salmonids. Within mammals, unique subpopulations of Ab-secreting cells (ASC) have been reported, i.e. plasmablasts and short- and long-lived plasma cells. Bromage et al. [66] presented evidence of ASC in trout (*Oncorhynchus mykiss*) immune tissue which was further reviewed by Ye et al [67]. In mammals, plasmablasts appear to dominate the early Ab response in peripheral immune organs, short-lived plasma cells (SLPC) are thought to be differentiated through clonal expansion of plasmablasts. Long-lived plasma cells (LLPC) may be generated by migration to a supportive niche in the bone marrow. LLPCs produce Ab for months to years without the stimulating Ag, relying on specialized cues. One suggested cue is type I IFN and

when injected as an adjuvant in mice it has been shown to induce both short- (10dpv) and long-lived (26wpv) Ab production [68]. It has been suggested that the signals type I IFN induce either affect migration to survival niches or differentiation of plasma cells [69]. Is it possible that CpG/polyI:C through its strong induction of type I IFN could enhance the generation of a, if present, similar long-lived Ab production in salmon?

4.2 Co-injection of vhsG with SAV Ag alone provided a slight increase in neutralizing responses while the opposite was evident for vhsG co-injection with SAV Ag CpG/polyI:C

The vhsG co-injected treatments did not provide any significant increase in protection. When vhsG was administered alone the protection was less or at the same level as for the saline group and the same trend was evident for SAV Ag Oil vhsG compared to SAV Ag Oil, which as suggested above could be due to a slower release of Ag. When analyzing the humoral responses, the SAV Ag Oil vhsG treatment interestingly showed no presence of (6wpv and 3wpc) or about 3/4 reduction (6wpc) in NHI titers compared to SAV Ag Oil. The water based SAV Ag formulation co-injected with vhsG had higher NT at 6wpv and 6wpc than SAV Ag alone. SAV Ag vhsG was the only vhsG co-injected group, except the SAV Ag CpG/polyI:C vhsG group that showed full protection at 3, 5 and 6 wpc. A reduction in neutralizing responses was present when vhsG was co-injected with the SAV Ag CpG/polyI:C formulation and the NHI NT were reduced by half or more at all three time points compared to SAV Ag CpG/polyI:C. These data suggests presence of factor(s) that provide a reduction in humoral responses when CpG/polyI:C adjuvanted SAV Ag is co-injected with vhsG. A similar reduction was not seen for SAV Ag vhsG, where a slight positive effect on humoral responses was present parallel to SAV Ag alone. Based on the presented data, an explanation for the opposing effect vhsG provide combined with the adjuvant and/or the Ag, cannot be provided without additional experiments. For example, it would be interesting to investigate presence of vhsG specific Ab in sera by ELISA, considering the notable increase in humoral responses seen in SAV Ag vhsG and the reduction seen for SAV Ag CpG/polyI:C vhsG. Further, to infect Atlantic salmon, vaccinated with the same treatments, with VHSV could indicate if antigen competition is responsible for the reduction in protective responses presented here. For SAV Ag CpG/polyI:C vs. SAV Ag CpG/polyI:C vhsG heat volatile humoral responses were the same pre challenge, with a titer of 640. Interestingly, SAV Ag CpG/polyI:C had the same titer for HI sera, indicating that CpG/polyI:C generates heat stable responses pre challenge. A decrease in (or same level) titers parallel to pre challenge titers were evident in the vhsG co-injected treatment. For HI

sera, vhsG co-injected SAV Ag CpG/polyI:C fish had same neutralizing responses (titer of 160) at all three samplings.

4.3 vhsG treatments showed a weak induction of IFN stimulated gene expression from 12 to 48 hpv in spleen

The vhsG co-injected treatments did not induce an additive or a synergistic impact on immune gene expression. Muscle samples (n=8) harvested 48 hpv from vhsG co-injected groups were analyzed for vhsG mRNA transcripts by a gene specific PCR (supplementary data). Transcription varied in the vhsG DNA vaccinated groups, with highest expression in the vhsG alone treated group (50%). A few –RT samples (reverse transcription reactions where the reverse transcriptase was omitted) indicated that vhsG DNA was present in the tissue. However, when adding reverse transcriptase the samples did not show any transcription. Therefore, the low transcription of vhsG in muscle and weak effect on immune genes could be explained by the relatively early sampling time point. McLauchlan et al. [70] has in rainbow trout shown that Mx is upregulated in liver 7 days post G DNA vaccination and in Japanese flounder (*Paralichthys olivaceus*) similar results has been presented [71] where Mx is upregulated 3 dpv in kidney, suggesting presence of a type I IFN response, known to induce upregulation of IFN stimulated genes (ISG). Martinez-Alonso et al. [72] showed that VHSV G protein injected i.m. in rainbow trout significantly upregulated Mx in spleen first 14 dpv (earlier samplings at 2 and 7 dpv did not show any expression) and IFNγ were not induced in spleen at any time point tested. Here, in all of the treatments (except CpG/polyI:C treated groups) IFNγ was modestly expressed in spleen, while its expression was more variable in HK. SAV Ag alone and SAV Ag Oil had the second highest expressions of IFNγ in HK, after treatments with CpG/polyI:C. For vhsG co-injected treatments IFNγ was at the same level or significantly down regulated parallel to SAV Ag alone in HK and spleen at both 12 and 48 hpv. Hypothetically, transcriptional responses against a certain stimulus depend on the leukocyte composition of the specific tissue analyzed [72, 73], suggesting a higher presence (or activity) of IFNγ secreting cells in groups without vhsG co-injection treatment in HK. Furthermore, expression of Mx and Vig-1 in vhsG treated groups increased from 12 to 48 hpv in spleen, both suggesting that the vhsG injection has had an effect on immune gene expression and an early type I IFN activation. However, significant upregulation of IFNa1 in spleen was detected first at 48 hpv in all vhsG treated groups compared to SAV Ag alone, demonstrating a later onset of IFNa1 expression in vhsG treatments. This makes it important to consider if other type I IFN subtypes were responsible

for the early induction of ISGs. Svingerud et al. [74] has shown that IFNb and especially IFNc are upregulated in spleen and HK after polyI:C stimulation.

4.4 vhsG does not contribute with any significant long term protective effects against SAV cohabitant challenge

To our knowledge, this is one of very few studies in fish that have been performed to investigate the protective effects of co-injecting an i.p. adjuvanted vaccine simultaneously with an i.m. injection of a DNA vaccine. A former study conjugated CpGs into a plasmid containing the G protein [72] studying vaccine induced immune responses. This study on rainbow trout, incorporated 2 or 4 CpG motifs into the plasmid backbone along with VHSV G protein, which gave significantly higher immune responses (Mx and IFNγ) and a significantly higher production of NAb compared to when plasmid without ODNs were administered [72]. Further, G DNA vaccination alone and subsequent viral challenge in rainbow trout has induced unspecific protection as early as 4 dpv against IHNV and 8 dpv for VHSV [53], which supports an early IFN related anti-viral response after i.m. injection of the glycoprotein. Protective immune responses displayed after challenge in G DNA vaccinated rainbow trout have been related to increased Mx expression and other ISGs [51, 52, 75], preceded by IFN induced responses through upregulation of type I IFN [76] and type II IFN [52]. This early unspecific protection has been followed by a more specific long lasting anti-viral response, based on both humoral (NAb) and cellular protective mechanisms (MHC II, T-cells) [75, 77, 78]. An early induced cross-protection after VHSV G protein vaccination has been seen following infection with nodavirus in turbot (*Scophthalmus maximus*) [54] and the VHSV heterologous virus IHN in rainbow trout, a study that also showed that the G protein does not confer protection against bacterial diseases [79]. Emphasizing that the early unspecific protection provided is anti-viral. As evident here, glycoprotein DNA vaccination has not contributed to any additive long lasting protective responses against SAV. In the same context, it would have been interesting to explore if the same vaccine combinations could have induced protective effects against a VHSV challenge.

To summarize, vaccination did not affect antiviral immune gene expression for vhsG treated groups at these early time points, but as indicated by Martinez-Alonso et al. [72], a later induction could be plausible. Pre challenge humoral responses for SAV Ag co-injected with vhsG had slightly higher levels of both heat volatile and heat stable neutralizing factors compared to SAV Ag alone, suggesting an additive effect of vhsG. Regarding CpG/polyI:C

treatments, no additive or synergistic effects were evident for either NHI or HI sera when co-injecting vhsG with the adjuvanted SAV Ag formulation. Instead, a rather negative effect was provided.

5 Conclusion

As shown here, CpG/polyI:C is a potent TLR-ligand combo for use in future salmonid vaccination strategies against SAV. There seems to be a very thin line between the SAV Ag dose conferring *some* protection, to the dose conferring *full* protection. Adding CpG/polyI:C as an adjuvant should be able to provide an Ag sparing effect, considering its potency. For the future – the need of knowing in detail how the interplay between innate and adaptive immune responses generates protection against disease in fish is still a key question for the field of fish immunology.

References

[1] M.D. Jansen, T. Taksdal, M.A. Wasmuth, B. Gjerset, E. Brun, A.B. Olsen, et al., Salmonid alphavirus (SAV) and pancreas disease (PD) in Atlantic salmon, *Salmo salar* L., in freshwater and seawater sites in Norway from 2006 to 2008, J Fish Dis, 33 (2010) 391-402.

[2] M. Crane and A. Hyatt, Viruses of fish: an overview of significant pathogens, Viruses, 3 (2011) 2025-2046.

[3] J.H. Weston, M.D. Welsh, M.F. McLoughlin and D. Todd, Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, Salmo salar L, Virology, 256 (1999) 188-195.

[4] E. Fringuelli, H.M. Rowley, J.C. Wilson, R. Hunter, H. Rodger and D.A. Graham, Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences, J Fish Dis, 31 (2008) 811-823.

[5] D.A. Graham, E. Fringuelli, H.M. Rowley, D. Cockerill, D.I. Cox, T. Turnbull, et al., Geographical distribution of salmonid alphavirus subtypes in marine farmed Atlantic salmon, Salmo salar L., in Scotland and Ireland, J Fish Dis, 35 (2012) 755-765.

[6] M.J. Hjortaas, H.R. Skjelstad, T. Taksdal, A.B. Olsen, R. Johansen, B. Bang-Jensen, et al., The first detections of subtype 2-related salmonid alphavirus (SAV2) in Atlantic salmon, Salmo salar L., in Norway, J Fish Dis, (2012).

[7] M.F. McLoughlin and D.A. Graham, Alphavirus infections in salmonids - a review, J Fish Dis, 30 (2007) 511-531.

[8] T. Crockford, F.D. Menzies, M.F. McLoughlin, S.B. Wheatley and E.A. Goodall, Aspects of the epizootiology of pancreas disease in farmed Atlantic salmon Salmo salar in Ireland, Dis Aquat Organ, 36 (1999) 113-119.

[9] A.B. Kristoffersen, H. Viljugrein, R.T. Kongtorp, E. Brun and P.A. Jansen, Risk factors for pancreas disease (PD) outbreaks in farmed Atlantic salmon and rainbow trout in Norway during 2003-2007, Prev Vet Med, 90 (2009) 127-136.

[10] S.K. Gahlawat, A.E. Ellis and B. Collet, A sensitive loop-mediated isothermal amplification (LAMP) method for detection of Renibacterium salmoninarum, causative agent of bacterial kidney disease in salmonids, J Fish Dis, 32 (2009) 491-497.

[11] E. Biering, S. Villoing, I. Sommerset and K.E. Christie, Update on viral vaccines for fish, Developments in biologicals, 121 (2005) 97-113.

[12] G. Houghton, Acquired protection in Atlantic salmon Salmo salar parr and post-smolts against pancreas disease, Dis Aquat Organ 18 (1994) 109-118.

[13] L. Desvignes, C. Quentel, F. Lamour and V.A. le, Pathogenesis and immune response in Atlantic salmon (Salmo salar L.) parr experimentally infected with salmon pancreas disease virus (SPDV), Fish Shellfish Immunol, 12 (2002) 77-95.

[14] N.R.T. McLoughlin M.F., Rowley H.M., Cox D.I. and Grant A.N, Experimental pancreas disease in Atlantic salmon Salmo salar postsmolts

induced by salmon pancreas disease virus (SPDV), Dis Aquat Organ, 26 (1996) 117-124.

[15] G. Houghton and A.E. Ellis, Pancreas disease in Atlantic salmon: serum neutralisation and passive immunisation, Fish Shellfish Immunol, 6 (1996) 465-472.

[16] M.V. Lopez-Doriga, D.A. Smail, R.J. Smith, A. Domenech, J. Castric, P.D. Smith, et al., Isolation of salmon pancreas disease virus (SPDV) in cell culture and its ability to protect against infection by the 'wild-type' agent, Fish Shellfish Immunol, 11 (2001) 505-522.

[17] M. Karlsen, T. Tingbo, I.T. Solbakk, O. Evensen, A. Furevik and A. Aas-Eng, Efficacy and safety of an inactivated vaccine against Salmonid alphavirus (family Togaviridae), Vaccine, 30 (2012) 5688-5694.

[18] C. Moriette, M. Leberre, A. Lamoureux, T.L. Lai and M. Bremont, Recovery of a recombinant salmonid alphavirus fully attenuated and protective for rainbow trout, J Virol, 80 (2006) 4088-4098.

[19] I. Sommerset, B. Krossøy, E. Biering and P. Frost, Vaccines for fish in aquaculture, Expert Review of Vaccines, 4 (2005) 89-101.

[20] S.P. Kasturi, I. Skountzou, R.A. Albrecht, D. Koutsonanos, T. Hua, H.I. Nakaya, et al., Programming the magnitude and persistence of antibody responses with innate immunity, Nature, 470 (2011) 543-547.

[21] A. Lahiri, P. Das and D. Chakravortty, Engagement of TLR signaling as adjuvant: towards smarter vaccine and beyond, Vaccine, 26 (2008) 6777-6783.

[22] H.R. Bohnenkamp, K.T. Papazisis, J.M. Burchell and J. Taylor-Papadimitriou, Synergism of Toll-like receptor-induced interleukin-12p70 secretion by monocyte-derived dendritic cells is mediated through p38 MAPK and lowers the threshold of T-helper cell type 1 responses, Cell Immunol, 247 (2007) 72-84.

[23] Q. Zhu, C. Egelston, A. Vivekanandhan, S. Uematsu, S. Akira, D.M. Klinman, et al., Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines, Proc Natl Acad Sci U S A, 105 (2008) 16260-16265.

[24] T. Querec, S. Bennouna, S. Alkan, Y. Laouar, K. Gorden, R. Flavell, et al., Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity, J Exp Med, 203 (2006) 413-424.

[25] F.M. Audibert and L.D. Lise, Adjuvants: current status, clinical perspectives and future prospects, Immunol Today, 14 (1993) 281-284.

[26] A.S. McKee, M.W. Munks and P. Marrack, How do adjuvants work? Important considerations for new generation adjuvants, Immunity, 27 (2007) 687-690.

[27] M. Singh and D.T. O'Hagan, Recent advances in veterinary vaccine adjuvants, International Journal for Parasitology, 33 (2003) 469-478.

[28] Ø. Evensen, Development in fish vaccinology with focus on delivery methodologies, adjuvants and formulations, Options Méditerranéennes, (2009) 177-186.

[29] A. Aunsmo, S. Ovretveit, O. Breck, P.S. Valle, R.B. Larssen and M. Sandberg, Modelling sources of variation and risk factors for spinal deformity in farmed Atlantic salmon using hierarchical- and cross-classified multilevel models, Prev Vet Med, 90 (2009) 137-145.

[30] E. Haugarvoll, I. Bjerkas, N.J. Szabo, M. Satoh and E.O. Koppang, Manifestations of systemic autoimmunity in vaccinated salmon, Vaccine, 28 (2010) 4961-4969.

[31] L.A. Skinner, P.M. Schulte, S.E. LaPatra, S.K. Balfry and R.S. McKinley, Growth and performance of Atlantic salmon, Salmo salar L., following administration of a rhabdovirus DNA vaccine alone or concurrently with an oil-adjuvanted, polyvalent vaccine, J Fish Dis, 31 (2008) 687-697.

[32] I. Skjæveland, D.B. Iliev, J. Zou, T. Jørgensen and J.B. Jørgensen, A TLR9 homolog that is up-regulated by IFN-[gamma] in Atlantic salmon (Salmo salar), Dev Comp Immunol, 32 (2008) 603-607.

[33] J.B. Jørgensen, A. Johansen, B. Stenersen and A.I. Sommer, CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (Salmo salar L.) leucocytes to produce supernatants with antiviral activity, Dev Comp Immunol, 25 (2001) 313-321.

[34] J.B. Jørgensen, L.-H. Johansen, K. Steiro and A. Johansen, CpG DNA Induces Protective Antiviral Immune Responses in Atlantic Salmon (Salmo salar L.), J Virol, 77 (2003) 11471-11479.

[35] Z. Meng, J. Shao and L. Xiang, CpG oligodeoxynucleotides activate grass carp (*Ctenopharyngodon idellus*) macrophages, Dev Comp Immunol, 27 (2003) 313-321.

[36] J.B. Jørgensen, J. Zou, A. Johansen and C.J. Secombes, Immunostimulatory CpG oligodeoxynucleotides stimulate expression of IL-1beta and interferon-like cytokines in rainbow trout macrophages via a chloroquine-sensitive mechanism, Fish Shellfish Immunol, 11 (2001) 673-682.

[37] A.C.M.A.R. Tassakka and M. Sakai, Expression of immune-related genes in the common carp (*Cyprinus carpio L.*) after stimulation by CpG oligodeoxynucleotides, Aquaculture, 242 (2004) 1-12.

[38] A.C. Carrington, B. Collet, J.W. Holland and C.J. Secombes, CpG oligodeoxynucleotides stimulate immune cell proliferation but not specific antibody production in rainbow trout (*Oncorhynchus mykiss*), Vet Immunol and Immunop, 101 (2004) 211-222.

[39] K.-y. Shen, C. Hogstrand and D.H. Davies, A newly identified CpG oligodeoxynucleotide motif that stimulates rainbow trout (*Oncorhynchus mykiss*) immune cells to produce immunomodulatory factors, Dev Comp Immunol, In Press, Corrected Proof (2006).

[40] A.C.M.A.R. Tassakka and M. Sakai, The in vitro effect of CpG-ODNs on the innate immune response of common carp, *Cyprinus carpio L.*, Aquaculture, 220 (2003) 27-36.

[41] D.B. Iliev, S.M. Jørgensen, M. Rode, A. Krasnov, I. Harneshaug and J.B. Jørgensen, CpG-induced secretion of MHCII[beta] and exosomes from salmon (Salmo salar) APCs, Dev Comp Immunol, 34 (2010) 29-41.

[42] A. Bagchi, E.A. Herrup, H.S. Warren, J. Trigilio, H.S. Shin, C. Valentine, et al., MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists, J Immunol, 178 (2007) 1164-1171.

[43] G. Napolitani, A. Rinaldi, F. Bertoni, F. Sallusto and A. Lanzavecchia, Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells, Nature Immunology, 6 (2005) 769-776.

[44] Y.S. Cheng and F. Xu, Anticancer function of polyinosinic-polycytidylic acid, Cancer Biol Ther, 10 (2011) 1219-1223.

[45] I. Jensen, A. Albuquerque, A.I. Sommer and B. Robertsen, Effect of poly I:C on the expression of Mx proteins and resistance against infection by infectious salmon anaemia virus in Atlantic salmon, Fish Shellfish Immunol, 13 (2002) 311-326.

[46] H. He, K.J. Genovese, C.L. Swaggerty, D.J. Nisbet and M.H. Kogut, In vivo priming heterophil innate immune functions and increasing resistance to Salmonella enteritidis infection in neonatal chickens by immune stimulatory CpG oligodeoxynucleotides, Vet Immunol Immunopathol, 117 (2007) 275-283.

[47] G. Strandskog, I. Skjaeveland, T. Ellingsen and J.B. Jørgensen, Double-stranded RNA- and CpG DNA-induced immune responses in Atlantic salmon: comparison and synergies, Vaccine, 26 (2008) 4704-4715.

[48] G. Strandskog, S. Villoing, D.B. Iliev, H.L. Thim, K.E. Christie and J.B. Jørgensen, Formulations combining CpG containing oliogonucleotides and poly I:C enhance the magnitude of immune responses and protection against pancreas disease in Atlantic salmon, Dev Comp Immunol, 35 (2011) 1116-1127.

[49] H.L. Thim, D.B. Iliev, K.E. Christie, S. Villoing, M.F. McLoughlin, G. Strandskog, et al., Immunoprotective activity of a Salmonid Alphavirus Vaccine: comparison of the immune responses induced by inactivated whole virus antigen formulations based on CpG class B oligonucleotides and poly I:C alone or combined with an oil adjuvant, Vaccine, 30 (2012) 4828-4834.

[50] N. Lorenzen and S.E. LaPatra, DNA vaccines for aquacultured fish, Rev Sci Tech, 24 (2005) 201-213.

[51] C.H. Kim, M.C. Johnson, J.D. Drennan, B.E. Simon, E. Thomann and J.A. Leong, DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish, J Virol, 74 (2000) 7048-7054.

[52] M.K. Purcell, K.M. Nichols, J.R. Winton, G. Kurath, G.H. Thorgaard, P. Wheeler, et al., Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus, Mol Immunol, 43 (2006) 2089-2106.

[53] N. Lorenzen, E. Lorenzen, K. Einer-Jensen and S.E. LaPatra, DNA vaccines as a tool for analysing the protective immune response against rhabdoviruses in rainbow trout, Fish Shellfish Immunol, 12 (2002) 439-453.

[54] I. Sommerset, E. Lorenzen, N. Lorenzen, H. Bleie and A.H. Nerland, A DNA vaccine directed against a rainbow trout rhabdovirus induces early protection against a nodavirus challenge in turbot, Vaccine, 21 (2003) 4661-4667.

[55] K. Hodneland and C. Endresen, Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan), J Virol Methods, 131 (2006) 184-192.

[56] M.W. Pfaffl, G.W. Horgan and L. Dempfle, Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, Nucleic Acids Res, 30 (2002) e36.

[57] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucl. Acids Res., 29 (2001) e45.

[58] C. Moriette, M. LeBerre, S.K. Boscher, J. Castric and M. Bremont, Characterization and mapping of monoclonal antibodies against the Sleeping disease virus, an aquatic alphavirus, J Gen Virol, 86 (2005) 3119-3127.

[59] S. Grove, L. Austbo, K. Hodneland, P. Frost, M. Lovoll, M. McLoughlin, et al., Immune parameters correlating with reduced susceptibility to pancreas disease in experimentally challenged Atlantic salmon (Salmo salar), Fish Shellfish Immunol, (2013).

[60] C.B. Brooke, A. Schafer, G.K. Matsushima, L.J. White and R.E. Johnston, Early activation of the host complement system is required to restrict central nervous system invasion and limit neuropathology during Venezuelan equine encephalitis virus infection, J Gen Virol, 93 (2012) 797-806.

[61] H. Grosfeld, B. Velan, M. Leitner, S. Cohen, S. Lustig, B.E. Lachmi, et al., Semliki Forest virus E2 envelope epitopes induce a nonneutralizing humoral response which protects mice against lethal challenge, J Virol, 63 (1989) 3416-3422.

[62] H. Grosfeld, S. Lustig, Y. Gozes, B. Velan, S. Cohen, M. Leitner, et al., Divergent envelope E2 alphavirus sequences spanning amino acids 297 to 352 induce in mice virus-specific protective immunity and antibodies with complement-mediated cytolytic activity, J Virol, 66 (1992) 1084-1090.

[63] M.M. Aasa-Chapman, S. Holuigue, K. Aubin, M. Wong, N.A. Jones, D. Cornforth, et al., Detection of antibody-dependent complement-mediated inactivation of both autologous and heterologous virus in primary human immunodeficiency virus type 1 infection, J Virol, 79 (2005) 2823-2830.

[64] S.M. Mangsbo, J. Sanchez, K. Anger, J.D. Lambris, K.N. Ekdahl, A.S. Loskog, et al., Complement activation by CpG in a human whole blood loop system: mechanisms and immunomodulatory effects, J Immunol, 183 (2009) 6724-6732.

[65] G. Strandskog, T. Ellingsen and J.B. Jørgensen, Characterization of three distinct CpG oligonucleotide classes which differ in ability to induce IFN alpha/beta activity and cell proliferation in Atlantic salmon (Salmo salar L.) leukocytes, Dev Comp Immunol, 31 (2007) 39-51.

[66] E.S. Bromage, I.M. Kaattari, P. Zwollo and S.L. Kaattari, Plasmablast and Plasma Cell Production and Distribution in Trout Immune Tissues, The Journal of Immunology, 173 (2004) 7317-7323.

[67] J. Ye, I. Kaattari and S. Kaattari, Plasmablasts and plasma cells: reconsidering teleost immune system organization, Dev Comp Immunol, 35 (2011) 1273-1281.

[68] A. Le Bon, G. Schiavoni, G. D'Agostino, I. Gresser, F. Belardelli and D.F. Tough, Type I Interferons Potently Enhance Humoral Immunity and Can Promote Isotype Switching by Stimulating Dendritic Cells In Vivo, Immunity, 14 (2001) 461-470.

[69] R.A. Manz, S. Arce, G. Cassese, A.E. Hauser, F. Hiepe and A. Radbruch, Humoral immunity and long-lived plasma cells, Current Opinion in Immunology, 14 (2002) 517-521.

[70] P.E. McLauchlan, B. Collet, E. Ingerslev, C.J. Secombes, N. Lorenzen and A.E. Ellis, DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection-early protection correlates with Mx expression, Fish Shellfish Immunol, 15 (2003) 39-50.

[71] J.Y. Byon, T. Ohira, I. Hirono and T. Aoki, Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (Paralichthys olivaceus) following DNA vaccination, Fish Shellfish Immunol, 18 (2005) 135-147.

[72] S. Martinez-Alonso, A. Martinez-Lopez, A. Estepa, A. Cuesta and C. Tafalla, The introduction of multi-copy CpG motifs into an antiviral DNA vaccine strongly up-regulates its immunogenicity in fish, Vaccine, 29 (2011) 1289-1296.

[73] J. Zou, A. Carrington, B. Collet, J.M. Dijkstra, Y. Yoshiura, N. Bols, et al., Identification and bioactivities of IFN-gamma in rainbow trout Oncorhynchus mykiss: the first Th1-type cytokine characterized functionally in fish, J Immunol, 175 (2005) 2484-2494.

[74] T. Svingerud, T. Solstad, B. Sun, M.L. Nyrud, O. Kileng, L. Greiner-Tollersrud, et al., Atlantic Salmon Type I IFN Subtypes Show Differences in Antiviral Activity and Cell-Dependent Expression: Evidence for High IFNb/IFNc-Producing Cells in Fish Lymphoid Tissues, J Immunol, 189 (2012) 5912-5923.

[75] P. Boudinot, M. Blanco, P. de Kinkelin and A. Benmansour, Combined DNA immunization with the glycoprotein gene of viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific response in rainbow trout, Virology, 249 (1998) 297-306.

[76] J. Zou, C. Tafalla, J. Truckle and C.J. Secombes, Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates, J Immunol, 179 (2007) 3859-3871.

[77] P. Boudinot, D. Bernard, S. Boubekeur, M.I. Thoulouze, M. Bremont and A. Benmansour, The glycoprotein of a fish rhabdovirus profiles the virus-specific T-cell repertoire in rainbow trout, J Gen Virol, 85 (2004) 3099-3108.

[78] N. Lorenzen and S.E. Lapatra, Immunity to rhabdoviruses in rainbow trout: the antibody response, Fish Shellfish Immunol, 9 (1999) 345-360.

[79] N. Lorenzen, E. Lorenzen, K. Einer-Jensen and S.E. LaPatra, Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens, Dev Comp Immunol, 26 (2002) 173-179.

Supplementary data

Expression of PcDNA-vhsG detected by PCR

Expression of vhsG mRNA transcripts was visualized by a gene specific PCR assay from muscle samples harvested 48 hpv and the transcription varied in the four vhsG DNA vaccinated groups; SAV Ag vhsG, SAV Ag Oil vhsG, SAV Ag CpG/polyI:C vhsG and vhsG alone, where vhsG mRNA had been transcribed in 14-50% of the muscle samples as specified in Table 1. The –RT samples (reverse transcription reactions where the reverse transcriptase was omitted) indicated that vhsG DNA was present in the tissue. However, when adding reverse transcriptase the samples did not show any transcription.

Table 1.

Number of individuals with PcDNA transcripts 48 hpv after gene specific PCR.

	Positive	Negative	Individuals (n)	Percent positive (%)	-RT
SAV Ag vhsG	1	6	7	14	-
SAV Ag Oil vhsG	3	5	8	37.5	-
SAV Ag CpG/polyI:C vhsG	2	6	8	25	3
vhsG	4	4	8	50	1
Saline	0	8	8	-	-