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Development of a nanoparticle-based oral vaccine for Atlantic salmon against ISAV using an alphavirus replicon as adjuvant

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ABSTRACT

Adjuvants used in vaccine aquaculture are frequently harmful for the fish, causing melanosis, granulomas and kidney damage. Along with that, vaccines are mostly administered by injection, causing pain and stress to the fish. We used the DNA coding for the replicase of alphavirus as adjuvant (Ad) of a vaccine against ISAV. The Ad and an inactivated ISAV (V) were loaded in chitosan nanoparticles (NPs) to be administered orally to Atlantic salmon. NP-Ad was able to deliver the DNA *ex vivo* and *in vivo*. Oral administration of the NPs stimulated the expression of immune molecules, but did not stimulate the humoral response. Although the vaccination with NP-V results in a modest protection of fish against ISAV, NP-V administered together with NP-Ad caused a protection of 77%. Therefore, the DNA coding for the replicase of alphavirus could be administered orally and can potentiate the immuneprotection of a virine against infection.

1. Introduction

Vaccination is widely used in aquaculture to keep the fish free from diseases. However, there are few cases of vaccines that have shown acceptable levels of protection [1,2]. Vaccines stimulate the immune protective response against pathogens and are often administered with adjuvants to increase the stimulation of the innate immune response of the host and strengthen the immune response stimulated by the antigen. However, in spite of the benefits they generate, the adjuvants used in aquaculture can cause adverse effects like the accumulation of myelin, tissue adherence, granulomatous peritonitis, growth inhibition, increased mortality [3–6], skeletal deformation [7], and even autoimmunity [8].

Alphavirus replicons have been shown to have major adjuvant capacity in mammals. It has been determined that the viral replicase complex in the cytoplasm of the cell can activate innate and specific immune response at various levels: interferon (IFN) [9–13], antibodies [14–17], and T cells [18,19]. This has allowed for the development of vaccines based on alphaviral replicons. One, the AVX701 (CEA(6D)-VRP) vaccine against cancer, which expresses a tumor carcinoembryonic antigen (CEA) packed in the replicon particle, is currently undergoing phase II clinical testing [9].

Recently, alphavirus replicons have been developed against Salmon Sleeping Disease (SSV) [20] and Pancreatic Disease (PD) [21]. These replicons have been used to develop a DNA vaccine against Infectious Salmon Anemia Disease (ISAV), which expresses hemagglutinin (HE), matrix or fusion protein as subgenomic RNA. It has been shown that the administration of inactivated ISAV, together with the DNA vaccine provides higher levels of protection than a virine alone administered by intramuscular injection [22]. This suggests that the product of DNA vaccine expression is an efficient adjuvant.

Abbreviations: NP, nanoparticle; Ad, adjuvant; V, virine.

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On the other hand, vaccines used in aquaculture are usually administered by injection. However, they are harmful to fish, causing melanosis, tissue adherence and increased mortality. Furthermore, administration by injection impedes the stimulation of the immune response similar to that activated by the pathogen. This is relevant, since fish have mucosa-associated lymphoid tissue (MALT) like, gut (GALT), skin (SALT) and gill (GIALT) that have significant immune response against pathogens [23]. In this respect, oral administration could be safer and activate similar immune response to the pathogen [23–25]. In particular, vaccines nanoencapsulated in chitosan deliver antigens in fish [26] and the expression of the encapsulated DNA in different organs [27–29]. This administration is capable of strengthening the immune system of the fish [30], generating a specific response and protection against antigens [28,31,32]. The characteristics and the degree of protection provided by vaccines in chitosan NPs depend on several factors, such as the size of the particles [33], the degree of cross-linking [34], and other physicochemical characteristics of the particles.

2. Methods

2.1. Reagents

Chitosan (low molecular weight; 75–85% deacetylated), sodium triphosphate pentabasic, and sodium acetate anhydrous for molecular biology ≥99%, were purchased from Sigma Chemicals (Sigma Co., St. Louis, MO); and sodium phosphate monobasic and sodium phosphate dibasic were purchased from Winkler Ltda. Plasmid DNA NSP-GFP [20], which contains the genes nsp1, nsp2, nsp3 y nsp4, codify for the viral replicase followed by the subgenomic region. In this plasmid, the subgenomic region, which in the alphaviral genome codifies for structural proteins, has been replaced for the sequence of green fluorescent protein (GFP). Thereby, GFP is expressed only if the viral replicase is expressed. This plasmid was kindly given by Dr. Michel Brémont from (INRA, Jouy en Josas, Francia), and was purified from nova blue bacteria and purified using the Maxi Kit NucleoBond® Xtra.

2.2. ISAV propagation and titration

The Atlantic Salmon Kidney (ASK) cell line was cultivated in T175 flasks at 16 °C in Leibovitz medium (L-15) supplemented with 10% fetal bovine serum (FBS), L-glutamine (6 mM), 2-mercaptoethanol (40 μM) and gentamycin (50 μg mL^{-1}). ASK cell monolayers were inoculated with a moi = 0.05 of the ISAV 752 strain in L-15 medium (without FBS) at 16 °C. Four hours later the cell culture was washed with PBS, and fresh L-15 medium was added.

The viral titer was determined as follows: The viral inoculum was diluted serially tenfold, in L15 medium (2×) without FBS and added to the cells. Four hours later the inoculum was removed and 3 mL of semi-solid medium was added to each well. The semi-solid medium was composed of L15 medium supplemented with FBS (10%), and LMP agarose (0.5%) (UltraPureTM Low Melting Point Agarose, Invitrogen). At 15 days post-infection the cells were fixed in 1 mL of formalin (37%) for 1 h at 25 °C, and the semi-solid medium was removed. For visualizing, 2 mL of crystal violet (1%) was added for 1 h at 25 °C, and finally the excess crystal violet was removed.

The ISAV genome was detected as described [35]. RNA was extracted using silica-based columns (EZNA®, Omega Biotek). RNA was quantified as copies/mL using primers described in Table 1. The qRT-PCR was performed in an Eco Real-Time PCR System (Illumina) using the commercial SensiMix SYBR One-step kit (Bioline) according to the manufacturer's specifications.

2.3. Viral inactivation

At 7 days post-infection (dpi), viral supernatants (10^6 copies/mL) were inactivated by UV light, heat or formaldehyde treatment. UV inactivation of ISAV was carried out irradiating with 4 or 9 mJ/cm^2 using an HL-2000 Hybrilinker-UV Crosslinker (UVP Laboratory Products) chamber at a volume of 15 mL of viral supernatant. Heat inactivation was carried out applying heat at 56 or 70 °C for 2 h. Incubating cells were treated with 0.02 or 0.2% formaldehyde for 3 h with magnetic stirring, followed by dialysis against PBS overnight. Inactivation of the virus was confirmed by viral RNA quantification and titration of ASK cells.

2.4. Anti-HE Western blot

Western blot was used to detect HE protein using mouse anti-HE 8D2/E9 (Grupo Bios/BiosChile) antibody at a 1:3000 dilution in TBS-T, and secondary goat HRP Anti-Mouse IgG (H + L) antibody (Invitrogen), at 1:7000 in TBS-T for 1 h at room temperature. The reaction was developed by chemiluminiscence.

2.5. Generation of recombinant EGFP

The EGFP sequence was amplified from the EGFP-N1 vector (Clontech), using the primers described in Table 1, and inserted in pGEM-T (Promega) according to the supplier's protocol, from which it was cut with NdeI/BamHI to insert it in pET15b (Novagen), which contained a histidine tag and was transformed into BL21. After the induction with IPTG, the bacterial pellet was resuspended in 40 mL of solubilization buffer (0.1% NP40, 10 mM imidazol, 300 mM NaCl, 50 mM pH 8.0 phosphate buffer, and complete Protease Inhibitor Cocktail Tablets (ROCHE). The bacteria were sonicated for 15 s and then incubated 1 min in ice, 5 times. The sonicated bacterial suspension was centrifuged at 7000 rpm in an SS-34 rotor (Sorvall) for 45 min at 4 °C, and the pellet was discarded. The 40 mL of crude extract and the Ni-NTA agarose resin (Biosonda Biotecnología, cat: 30210) were incubated at 4 °C with constant stirring. The mixture was centrifuged at 3000 rpm, washed with 10 mL of washing buffer (0.1% NP40, 25 mM imidazol, 1 M NaCl, 50 mM pH 8.0 phosphate buffer, 10 mM β-mercaptoethanol, 20% glycerol), centrifuged, deposited on a 20 mL column, and washed with 2 mL of washing buffer. The elution was performed with elution buffer 1 (0.1% NP40; 50 mM imidazol; 1 M NaCl; 50 mM pH 8.0 phosphate buffer, 10 mM β-mercaptoethanol, 20% glycerol), followed by elution buffer 2 (0.1% NP40; 300 mM imidazol; 1 M NaCl; 50 mM pH 8.0 phosphate buffer, 10 mM β-mercaptoethanol, 20% glycerol), yielding four 1-mL fractions from each elution. The buffer of the chosen eluates was changed (150 mM NaCl, 50 mM pH 8.0 phosphate buffer, 20% glycerol) in Amicon columns (Amicon® Ultra 4 mL Filters for Protein Purification and Concentration, Merck millipore), according to the protocols of manufacturer. The fractions were analyzed by Western blot to detect the histidine tag with a histidine anti-tag antibody (anti-His-HRP monoclonal antibody, Sigma–Aldrich)) 1:3000 under the same conditions as the Western anti-HE.

2.6. Preparation of the chitosan NPs

The NPs were made by the ionic gelation method, using described protocols [36,37] with modifications (Table 2). The pellet was resuspended in water. The encapsulation efficiency was determined by quantifying protein, DNA or virine in the supernatant by the Bradford assay, absorbance at 260 nm, or qRT-PCR,

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Table 1
Primers used in this investigation.

Primer	Sequence	Genbank accession number	Reference
ISAV-F	GAAGAGTCAGGATGCCAAGACG		[67]
ISAV-R	GAAGTCGATGAACTGCAGCGA		
GFP-F	CATATGGTGAGCAAGGGCGA	U55762	Self design
GFP-R	GGATCCTTACTTGTACAGCTCGTCCATGC		
18S-F	CTTAGATGTCCGGGGCT	FJ710874.1	Self design
18S-R	CTCGGCGAAGGGTAGACA		
INFα-R	GGGCGTAGCTTCTGAAATGA	AY216595	[66]
INFα-F	CGTCATCTGCAAAGATTGGA		
INFγ-R	TGTACTGAGCGGCATTACTCC	AY795563	[66]
INFγ-F	AAGGGCTGTGATGTGTTTCTG		
IL10-R	TGTTTCCGATGGAGTCGATG	EF165029	[66]
IL10-F	GGGTGTCACGCTATGGACAG		
TGFβ-R	AGCTCTCGGAAGAAACGACA	EU082211	[66]
TGFβ-F	AGTAGCCAGTGGGTTCATGG		
CD4-R	CCCCGACTCCGCCCATCTCA	AY973030.1	Self design
CD4-F	CCGCTGTCTGTGGCGTCGGTT		
IL-12 F	GAGCCAAGTCTTATGGCTGC	BT049114	Self design
IL-12 R	GTTCAAACTCCAACCCTCCA		

Table 2
Inactivation of ISAV.

	Inactivation							
	Negative control	Mock	UV (mJ/cm^2)		Heat (°C)		Formaldehyde (%)	
			4	9	56	70	0.02	0.2
Inactivation percentage	99.99	–	0	98.12 ± 3.26	0	43.98 ± 97	20.95 ± 4.4	99.98 ± 0.01
Plaques of lysis	0	10^8	–	0	10^8	–	8×10^5	0

respectively, according to the formula: %E= (total amount counterion −free amount counterion)/(total amount counterion) × 100.

2.7. Physicochemical characterization of the NPs

The chitosan nanoparticles were characterized by low-voltage electron microscopy. Briefly, one drop of diluted aqueous solution was placed on an ultra-thin Lacey carbon-coated 400-mesh copper grid and dried for 10 min prior to image acquisition. An LVEM5 electron microscope (Delong Instruments, Montreal, Quebec, Canada) at a nominal voltage of 5 kV was used to acquire TEM images, which were captured using a Retiga 4000R camera (QImaging, Inc., USA) at its maximum resolution. The use of low voltage ensured a low electron dose on the sample and delivered high contrast without heavy-metal staining procedures, allowing the direct visualization of soft-materials with decreased sample damage compared to high-voltage electron microscopy.

2.8. Transfection of SHK-1 cells with NPs

SHK-1 cells grown on coverslips were incubated with chitosan NPs encapsulating the DNA at a concentration of 50 NP-Ad cell in L-25 medium supplemented with 10% SFB. At twenty-four hours post-incubation, the cells were washed with PBS and fresh medium was added. GFP expression was evaluated in an epifluorescence microscope Olympus BX40, twenty-four hours later.

2.9. Determination of the expression of NPs in the fish

Ten fish Atlantic salmon (*Salmo salar*) weighing 40 g were kept in fresh water tanks at a density of 22 kg/m^3, a temperature of 14–18 °C, and an oxygen rate of 5.8–7.1 mg/L. After 15 days of acclimatization, fish were inoculated by intramuscular injection with 100 μL of NP-Ad containing 3 μg of DNA NSP-GFP per fish. After 4 days, 3 fish were sacrificed by immersing them in excess of benzocaine. The expression of the adjuvant in the intestine of vaccinated fish was determined as follow: portions of muscle of injected fish were fixed in 4% paraformaldehyde in a 0.1 M PBS solution for 2 days, and then transferred to a 30% (w/v) sucrose/PBS solution containing 0.01% (w/v) sodium azide. Tissue samples were embedded in tissue tek (4583, Sakura) and cut in a microtome Bright Starlet 2211 BioJSP into 5 μm-thick sections. The sections were mounted on coverslips and examined in a confocal microscope (Carl Zeiss, LSM510).

2.10. Oral administration of NP and sampling

To prepare the mixture of vaccines NP-V, NP-Ad or NP-Ad + NP-V were mixed homogeneously with the fish feed (Alimentos Pilar SA, Buenos Aires, Argentina) with a dose of 7 μg of DNA and 1×10^5 $TCID_{50}$ of virine per fish. The feed was dried for 1 h at 30 °C, and covered with 2% vegetable oil. Three groups of 10 fish Atlantic salmon (see point 2.9) were feeded with vaccine preparation in a proportion of 1% of the fish weight for 7 days. Group 1 was vaccinated with NP-V, group 2 with NP-Ad, and group 3 with NP-Ad + NP-V. On day 4 post-treatment 3 fish per group were sampled and the expression of molecular marker was evaluated by real time PCR.

2.11. Real time PCR

Total RNA from spleen, kidneys, gills and gut were extracted with Trizol (Invitrogen) according to the manufacturer's protocol. The expression of mRNAs 18S, CD4, INFα, INFγ, IL-10, IL-12 and TGFβ was quantified using Brilliant SYBR® Green Q-RTPCR Master Mix (Stratagene) according to the manufacturer, using previously described primers (Table 2). The primer concentration was 0.5 μM for each primer and the thermal profile used was 10 min at 95 °C,

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40 cycles of 15 s at 95 °C, 15 s at 58 °C, and 30 s at 72 °C. Gene expression was quantified according to the Pfaffl mathematical model [38] using 18S messenger quantification as a reference gene. Immune molecules expression was evaluated for statistically significant differences by the ANOVA and Bonferroni test. $p < 0.05$ was considered a significant change.

2.12. Challenge test

Four groups of 30 fish Atlantic salmon (*S. salar*) with an average weight of 70 g were kept in fresh water tanks at a density of 22 kg/m^3, a temperature of 14–18 °C, and an oxygen rate of 5.8–7.1 mg/L. After 15 days of acclimatization, group 1 was vaccinated with NP-V, group 2 was vaccinated with NP-Ad + NP-V, and groups 3 and 4 were fed with food without NP. On day 7 post vaccination, 3 fish per group were sampled to evaluate the expression of GFP in the gut as explained above (2.9). Some intestine samples were stained with propidium iodide (10 μg/mL in PBS) for 2 min and then washed with PBS twice in the coverslip, before the examination by confocal microscopy.

After 450 UTA, 3 fish per group were sampled to determine the IgM antibody response (see below). After that, all fish of groups 1, 2 and 3 were challenged with an intraperitoneal injection with 1×10^5 $TCID_{50}$ of ISAV. The overall condition of the fish was verified and the number of deaths due to the infection was determined. Relative percentage survival (RPS) was determined as, RPS= (1- % mortality in vaccinated/% mortality in control fish) x 100.

2.13. Evaluation of the antibody response by ELISA

The antibodies contained in cutaneous mucus, intestinal mucus, bile, gill and blood serum were evaluated by ELISA. Dorsal cutaneous mucus was removed carefully from anesthesized with 1x benzocaine fish by scraping with a flat spatula between the head and the dorsal fin, depositing the mucus in a solution of 200 μL of PBS + 1x protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche). Blood was removed by caudal puncture from fish. After sacrificing by benzocaine overdose, the second and third gills were extracted, the gill arches were discarded and the complete filaments were deposited in 200 μL PBS + 1x protease inhibitor solution. The proximal portion of the intestine was extracted and opened, cleaning away fecal remains. The mucus was scraped with a scalpel into another 200 μL of PBS + 1x protease inhibitor. Finally, the complete bag containing the bile was deposited in PBS + 1x protease inhibitor. These suspensions were centrifugated at 8000 × g during 10 min and the supernatant was storaged at −20 °C. Elisa assay was performed as follow: 96 well plate (Nunc™ MaxiSorp™, Thermo Scientific) was adsorbed with 1.0 μg per well of total protein of ISAV, in 100 μl of 0.2 M sodium carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4 °C. Wells were washed 3 times with PBS 0.05% Tween 20 (T-PBS) and blocked by adding to each well 200 μl of Tris–HCl 30 mM pH 8.0, NaCl 140 mM, bovine serum albumin 1% w/v at room temperature for 1 h. The blocking buffer was removed and the plates were washed as described above. Fish serum samples in 2-fold dilutions (from 1:4 to 1:1024) made in blocking buffer were added to the wells (100 μl per well) in triplicate and incubated at room temperature for 1 h. Also, mucus from gill, gut, skin and billis was incubated without dilution in the plate. Blank (PBS-T), positive (serum from infected fish) and negative control (serum from non-infected fish) was evaluated. Later, the plate was washed 3 times, and incubated with 100 μl of 1:100 dilution in blocking buffer of Anti-IgM of salmon IgG fraction monoclonal antibody (clone 3H7/E1, Grupo Bios/BiosChile), during 1 h at room temperature. After 3 washes, plate was incubated with a dilution of 1:3000 of IgG goat anti-mouse-HRP (Dako Denmark) in blocking buffer. The plates were washed 3 times and 50 μl of HRP substrate ABTS-H_20_2 was added and incubated during 20 min in darkness. The reaction was stopped by addition of 50 μL sulphuric acid 1 M and O.D. was determined at 415/405 nm in an ELISA reader modelo 550, Multiskan (Bio-Rad).

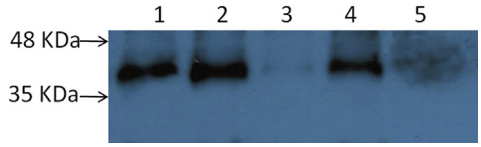

Fig. 1. Western-blot against HE in inactivated ISAV. 1. Partially purified wild-type ISAV; 2. Supernatant of infected cell culture. 3. Treatment with 0.2% formaldehyde. 4. Treatment with UV 9 mJ/cm^2. 5. Treatment with at 70 °C. Molecular weight of AccuRuler RGB Prestained Protein Ladder (Maestrogen) are indicated.

3. Results

3.1. Inactivation of ISAV

To obtain the antigen of the vaccine, we prepared inactivated viral particles (virine) of ISAV. Three inactivation methods were tested: i) UV, ii) heat, and iii) incubating with formaldehyde. The inactivation of the ISAV was verified by the absence of infection after three successive passages in ASK cells by viral RNA detection and viral titer by lysis plaque. To determine whether the technique of inactivation had not modified the viral epitopes in the virine, specific antibodies were used to detect the viral HE protein by Western blot. HE has been reported as the most antigenic of the ISAV proteins [39]. This experiment (Table 2) showed that the treatment with 0.2% formaldehyde and UV at 9 mJ/cm^2 inactivated the virus, while the application of heat did not totally inactivate the ISAV, even at high temperature. Only UV was capable of inactivating the virus while maintaining the immunogenicity of the HE protein (Fig. 1), therefore, ISAV inactivated by UV radiation was selected as the antigen of the vaccine.

3.2. Nanoencapsulation

Enhanced green fluorescent protein (EGFP) was used to implement the technique of nanoencapsulation. This protein was obtained by expression in BL21 bacteria in large amounts using a methodology developed in this investigation (Fig. 2). Different conditions to obtain the EGFP nanoparticle (NP-EGFP) were evaluated (Table 3). The encapsulation technique that achieved 80–90% of encapsulation used 2 mg/mL chitosan in 1 M acetate buffer at pH 5.0; 1 mg/mL of TPP 1 M phosphate buffer at pH 8.0, and a chitosan:TPP ratio of 5:1. The drip flow was 0.3–0.6 mL/min, and stirring at 800 rpm. After formation, the nanoparticles were stirred for 30 min and centrifuged for 1 h at 10,000 rpm. This protocol was used for the nanoencapsulation of the virine (NP–V) and DNA of the adjuvant that encodes for,-GFP (NP-Ad). The efficiency of the

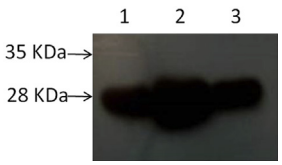

Fig. 2. Preparation of recombinant EGFP. Western blot using anti-His (Sigma) of EGFP-Hys obtained by expression in BL21 cells. 1, 2 and 3 represent three purification-by-elution fractions. Molecular weight of AccuRuler RGB Prestained Protein Ladder (Maestrogen) are indicated.

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Table 3
Conditions for generating nanoparticles.

Condition	Variable	Nanoparticles obtained	% of loading efficiency
Solubilization 40 mg chitosan/20 mL of acetate buffer	0.4 M pH 6.0	No dissolution	–
	0.4 M pH 5.5	No dissolution	–
	0.1 M pH 5.5	No dissolution	–
	0.1 M pH 5.0	–	–
Solubilization 1 mg/mL TPP	Water	+–	<10
	Buffer Phosphate 0.1 M pH 8.0	+	>80
Dropping (TPP + GFP) into chitosan solution	**0.3–0.6 mL/min**	+	80–90
	>1 mL/min	+	70–80
	>5 mL/min	+	<10
Dropping (chitosan + GFP) into TPP solution	0.3–0.6 mL/min	+–	<10
Stirring time	10 min	+	75–80
	30 min	+	80–90
Centrifugation time	30 min at 10,000 rpm	+	70–80
	60 min at 10,000 rpm	+	80–90

The conditions outstandings in bold were selected to obtain the nanopartiles used in this investigation.

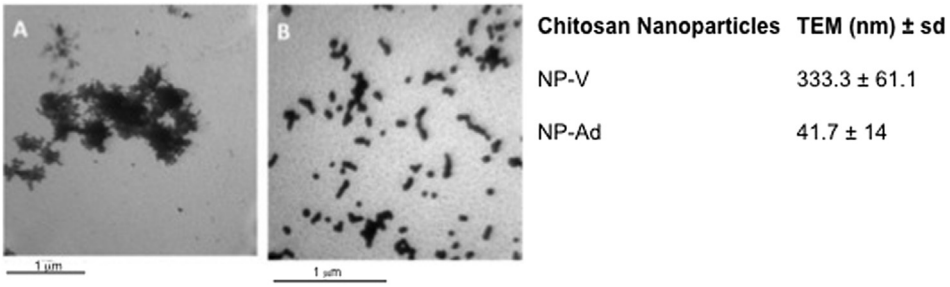

Fig. 3. Scanning electron microscopy of the nanoparticles A: NP-Ad. B: NP-V. Inserted table show size of the NPs determined by electron microscopy. Sd = standard deviation.

nanoencapsulation was determined by qRT-PCR and absorption spectroscopy at 280 nm, respectively, yielding an encapsulation efficiency of 85.6 ± 4.9% for NP-V and 86 ± 1.73% for NP-Ad.

3.3. Characterization of the nanoparticles

The NPs obtained by this procedure were analyzed by scanning electron microscopy (Fig. 3). Fig. 3A shows that the NP-Vs had a diameter of around 300 nm, a heterogeneous and rounded morphology, with a high electron density in the center of the nanoparticle of approximately 200 nm in diameter, surrounded by an area of low electron density, depending on the kind of material. The NP-Ad (Fig. 3B) had a diameter of around 40 nm, with a higher electron density, and were more rounded and uniform.

The release of the load of the NPs and the expression of the adjuvant were verified *ex vivo* and *in vivo*. For this purpose, SHK-1 cells were incubated with the NP-Ad. Fig. 4A shows that the SHK-1 cells express GFP encoded in the encapsulated NSP-GFP. The expression of the adjuvant *in vivo* was verified in fish four days after the intramuscular injection of 3 μg of NP-Ad (Fig. 4B), showing that the NPs are capable of releasing their load and allowing the expression of the DNA in the fish.

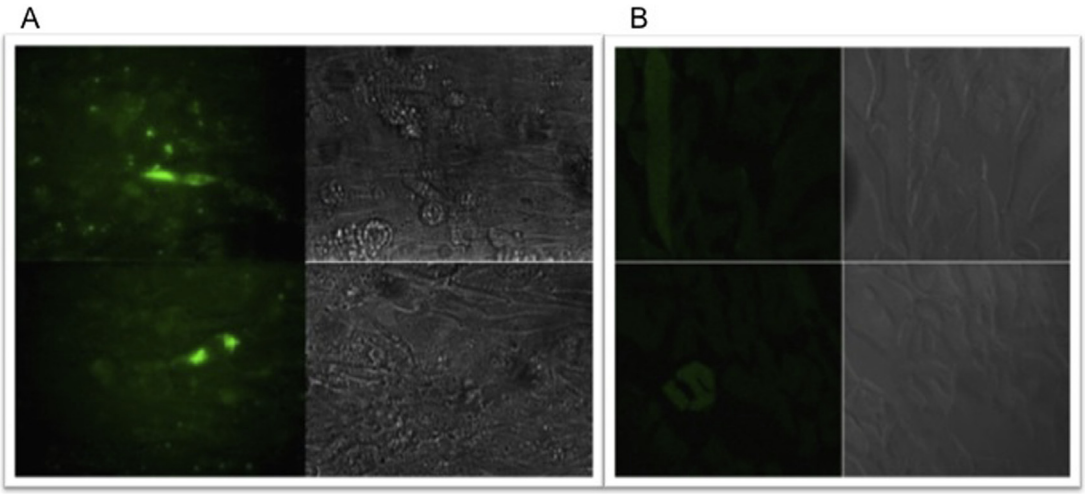

Fig. 4. Expression of the DNA encapsulated in NP. A: Expression of the GFP coded in NP-Ad *ex vivo* SHK-1 cells were incubated with NP-Ad in a 1:50 ratio for 48 h. Epifluorescent and phase contrast images of the coveslip are shown. B: Expression of the GFP coded in NP-Ad *in vivo*. Atlantic salmon injected with NP-Ad were sacrificed at 4 days. Muscle sections were fixed, cut in a microtome, and examined by confocal microscopy.

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3.4. Evaluation of the effect of the oral administration of the NPs on the immune response of Atlantic salmon

To evaluate the effect of NPs on immune response of Atlantic salmon, the NPs were administered orally to 40 g Atlantic salmon for 7 days. For this purpose, NP-V, NP-Ad or NP-V + NP-Ad were mixed with the fish feed, in a dose of 1×10^5 $TCID_{50}$ of NP-V and 7 μg of DNA in NP-Ad per fish. The levels of expression of the immune molecules IFNα, IFNγ, IL-10, TGF-β, IL-12 and CD4 in the spleen, kidneys, intestine and gills were determined at 4 days after administration (Fig. 5). Antiviral IFNα and IFNγ cytokines were highly stimulated: IFNα was stimulated significantly by NP-V (175 times) and NP-Ad (182-fold) in the spleen, and in the kidneys (30- and 87-fold), intestine (84- and 38-fold) and gills (25- and 141-fold). The administration of NP-V + NP-Ad strengthened the increase in the kidneys, intestine and gills, reaching stimulations of 561-, 121- and 256-fold, respectively. IFNγ was stimulated in the gills, reaching 3.6-fold by NP-V and 13.6-fold by NP-Ad. The stimulation in the intestine was six-fold by NP-V and three-fold by NP-Ad. Although IFNγ in the spleen and the kidneys was only slightly stimulated by NPs administered separately (1.7 and 0.7 for NP-V and 0.8 and 1.4 for NP-Ad), the mixture NP-V + NP-Ad caused an increased response of 2.8- and 5-fold, respectively. In the gills, the NP-Ad alone and mixed with NP-V stimulated IFNγ around 14 times.

The regulatory cytokines IL-10 and TGF-β were more stimulated in the kidneys by the administration of NP-V + NP-Ad (12 and 22 times, respectively) than by the two separately (0.8 and 2.28 by IL-10, and 3.8 and 9.4 by TGF-β). NP-V induced IL-10 to increase by 5 times and TGF-β by 19 times in the intestine, which did not occur

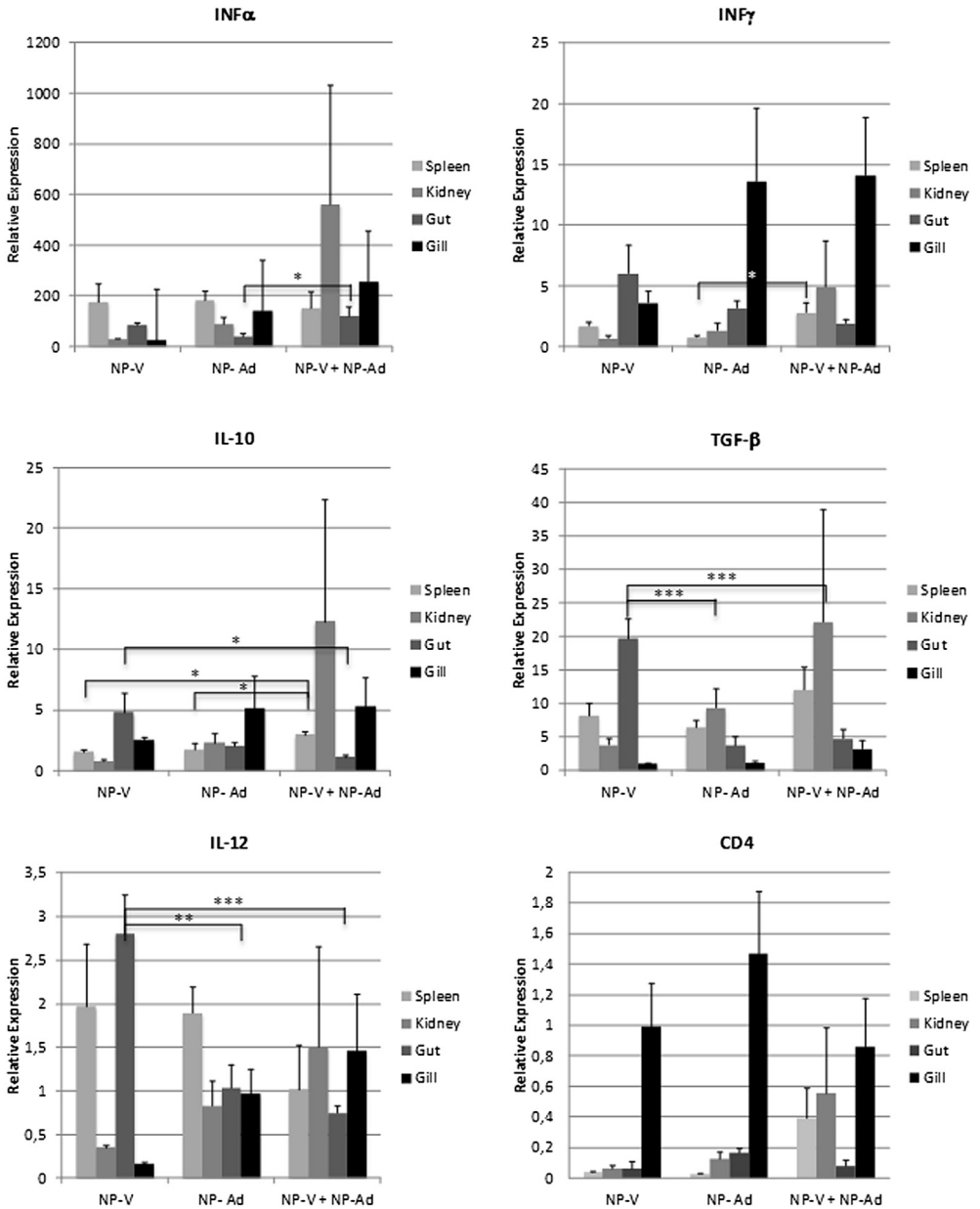

Fig. 5. Effect of the oral administration of NP-V, NP-Ad or NP-V + NP-Ad on cytokine expression. Fish were fed with food mixed with NPs or not for 7 days. At 4 days post-vaccination, the levels of expression of IFNα, IFNγ, IL-10, TGF-β, IL-12, and CD4 were determined in the spleen, kidneys, intestine and gills of Atlantic salmon. Immune molecules expression was evaluated for statistically significant differences by the ANOVA and Bonferroni methods. $P < 0.05$ was considered a significant change. *$p < 0.05$; ** $0.0001 < p < 0.05$; ***$p < 0.0001$.

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when NP-V + NP-Ad were administered. In the gills, NP-Ad caused a 5-fold increase of IL-10 regardless of the administration of NP-V. Finally, IL-12 and CD4 did not show a substantial increase for the NP-V + NP-Ad mixture, indicating that the vaccine does not have a role in stimulating the immune cell response. Even so, the administration of NP-V did cause a 2.8-fold increase of IL-12 in the intestine, but this did not occur when NP-Ad was added. These results show that the vaccine is capable of stimulating the innate immune response through IFNα, and the immune cell response through IFNγ, and that the stimulus of this response can be regulated by the stimulation of IL-10 and TGF-β.

We evaluated the humoral response of the antibodies in the blood, bile and cutaneous, intestinal and gill mucus at 30 days post-NP-administration, and found no stimulation of antibody secretion at any level (data not shown).

3.5. Protection by the vaccine against ISAV infection

The ability of vaccinations with NP-V or the NP-V + NP-Ad mix to protect Atlantic salmon was determined by ISAV challenge. Fish at 70 g were vaccinated orally as indicated above for 7 days. The expression of the DNA in the intestine was determined at 7 days post-vaccination (Fig. 6). After 450 UTA, fish were challenged with 1×10^5 $TCID_{50}$ of ISAV per fish. Fig. 7 shows that mortality began at 9 days post-infection, reaching 48% in the positive control on day 25 post-infection. The administration of NP-V allowed obtaining a 29% of cumulative mortality, protecting 40.4%. However, the vaccination with the mixture NP-V + NP-Ad reduced mortality more significantly, reaching the maximum protection of 77.9%, showing that vaccines based on virines plus the adjuvant encapsulated in chitosan are capable of providing protection to Atlantic salmon when administered orally.

4. Discussion

This study assessed a new type of adjuvant based on the DNA coding for replicase of SAV and the GFP coded by the subgenomic RNA. This adjuvant was administered orally in chitosan NPs with an ISAV virine.

There are documented strategies to inactivate ISAV: ie. 0.33 mJ/cm^2 [40,41]; 7.7 mJ/cm^2 [42]; 56 °C [41]. However, in our hands, only more aggressive treatments with formaldehyde 0.2% and UV 9 mJ/cm^2 inactivated the virus completely, while heat does not inactivate it completely. The treatments were also much more aggressive than those used to inactivate the influenza virus, of the same family as ISAV [22,43,44]. These results may indicate that different ISAV isolates have different susceptibility to inactivation conditions. In addition, only the treatment with UV radiation maintained the immunogenicity of HE by Western blot using an antibody directed against the wild virus. The development of an immunogenic ISAV virine is extremely important, because it has been found that ISAV virines are not highly immunoprotective [45]. This could be because the inactivation process can disrupt wild epitopes in the inactivated virus, diminishing the immunogenicity of the virine. It was expected that inactivation with formaldehyde and heat would disturb the structure of the viral proteins, and that UV radiation would not, since it mostly affects nucleic acids, causing crosslinking between them and nearby proteins.

The virine and adjuvant were loaded with chitosan, yielding two types of NPs. The first is NP-V, which is the larger, with a diameter

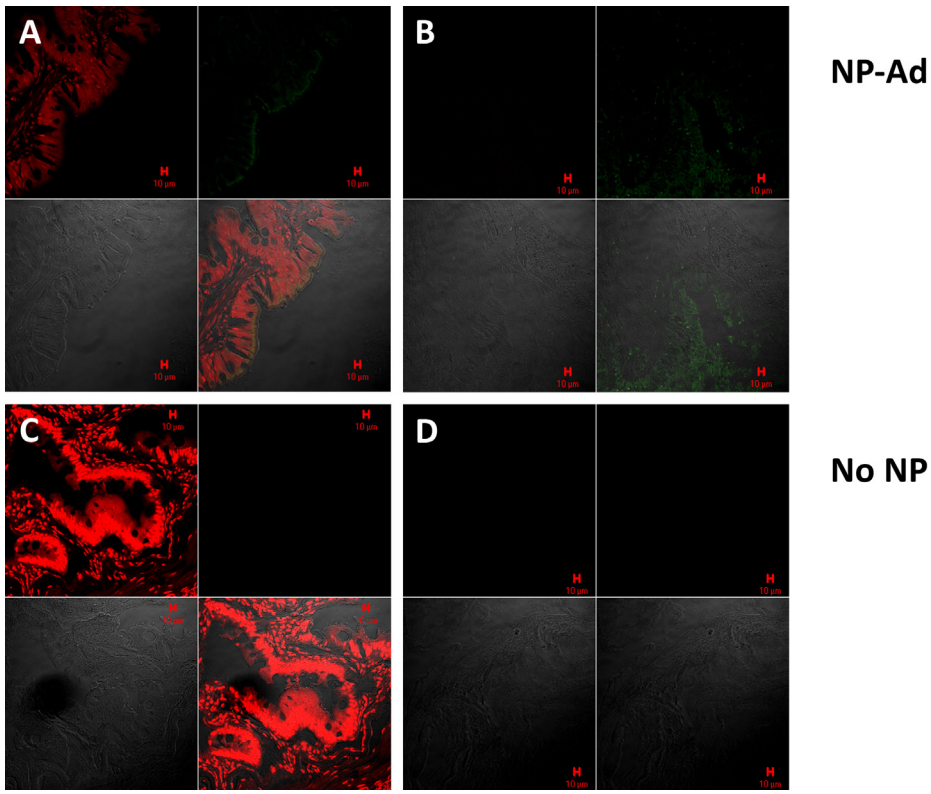

Fig. 6. Expression of the adjuvant in the intestine of vaccinated fish. Fish were vaccinated orally with NP-Ad + NP-V. At 7 days post-vaccination the expression of the adjuvant was determined in the intestine. Upper panel: vaccinated fish. Lower panel: control fish. Tissue samples were incubated with propidium iodide (A and C) or not (B and D).

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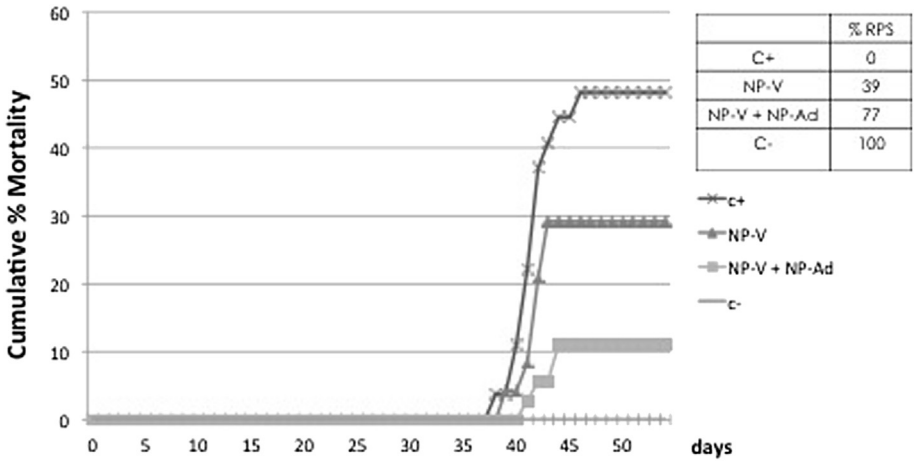

Fig. 7. Atlantic salmon vaccinated with NPs are protected against ISAV. Fish were fed with food without NP or food mixed with NP-V, NP-V + NP-Ad for 7 days. At 30 days fish were challenged with ISAV. Cumulative mortality was determined daily. The table shows the RPS at the end of the experiment.

of around 300 nm. Its core, which is more electron-dense than the surface, is approximately 40 nm in diameter, which is similar to the diameter of ISAV. It suggests that the virine is in the middle of the NP and surrounded by chitosan, NP-Ad is the smaller and more compact of the two NPs, suggesting that the different DNA and chitosan charges allowed for the formation of a dense NP. The encapsulation of DNA has been documented extensively in mammals [46], and there are also some reports in fish [27–29,32]. The small size of the prepared NPs could allow them to be incorporated by cells [47–49]. In fact, the NPs were able to transfect fish cells *ex vivo* and *in vivo*, ensuring that their administration generates the expressed protein in the fish.

The NPs produced in this work were able to prevent ISAV infection in Atlantic salmon. NP-V provided 40.4% protection. The vaccination with the mixture NP-V + NP-Ad mixture provided 77.9% protection. The adjuvant was able to significantly increase the protection provided by the virine against ISAV. This experiment has also shown that vaccines based on virines plus adjuvant encapsulated in chitosan are capable of providing protection to Atlantic salmon when administered orally. These results are very important given that other reports have found that vaccination with chitosan microspheres provides little protection when administered orally (Rajesh Kumar, Ishaq Ahmed et al., 2008) and that the administration of nanoencapsulated vaccines to fish in general are not highly immunoprotective [50].

It was expected that the vaccine administered orally would stimulate the immune response from various fronts in the fish, as does the pathogen, and that the expression of the DNA in the cells would allow the stimulation of an antiviral immune response [50], causing a robust response against the pathogen. Indeed, the antiviral response was highly stimulated by NP-V and NP-Ad, with a synergic effect on IFNα expression in the kidneys, intestine and gills, and IFNγ in the kidneys when NP-V + NP-Ad were administered. Strengthening of the response when both NPs were administered was also observed in IL-10 in the kidney, and TGF-β in the spleen, kidneys and gills, confirming the adjuvant ability of the alphavirus replicase. The kidney and spleen are the most important immune organs in fish, with the kidneys considered as the primary and the spleen the secondary organ [51–54]. As well, the primary response against a pathogen takes place where it is met: the gills and intestine. The gills, together with the skin, are the first barriers against the entry of pathogens. The vaccine was capable of stimulating a powerful antiviral response in the gills, reaching 256-fold for IFNα and 14.1-fold for IFNγ. The antiviral response stimulated in the intestine was also notable, reaching 121-fold for IFNα and 2-fold for IFNγ. The antiviral response was also stimulated in the spleen and the kidneys, reaching 150-fold and 561-fold, respectively, for IFNα, and 2.8-fold and 5-fold, respectively, for IFNγ, arguably a notable level of protection against the pathogen.

The antiinflammatory response was also stimulated in the gills and intestine, reaching 5.2-fold and 1-fold, respectively, for IL-10, and 3.2-fold and 4.8-fold, respectively, for TGF-β, while in the spleen and the kidneys it was 2.9-fold and 12-fold, respectively, for IL-10, and 12-fold and 22-fold, respectively, for TGF-β. This shows that the regulation of the immune response was more marked in the latter organs.

Finally, although there was no major response in CD4 or IL-12, indicating that there was no important cell response, the increased response of IFNγ, and the lack of measurements of other cytokines do not rule out that the immune cell response is involved in protecting against the pathogen. In addition, to achieve the cellular specific immune response it could be necessary to measure the cytokine expression at longer time scales.

There was no antibody stimulation in the blood, cutaneous, gill or intestinal mucus, or bile, showing that the oral administration of the NPs was not adequate to stimulate antibody response. Some reports link the protection of vaccines against pathogens with their ability to stimulate antibodies present in mucus rather than antibodies in the blood [23,24,55,56]. However, some studies state that protection from the pathogen does not correlate strongly with the stimulation of the humoral immune response in the blood [45,57–62], and results related with the stimulation of IgM in mucus by oral vaccination are in contradiction [63–65].

In conclusion, in this work we have demonstrated the adjuvant capacity of the DNA that codes for the SAV replicase of alphavirus. The production of a vaccine nanoencapsulated in chitosan, together with DNA as adjuvant, can be administered to salmon orally in feed to protect against ISAV. This opens new possibilities in the development of protective vaccines that are not invasive or harmful to fish.

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