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An oral DNA vaccine against infectious haematopoietic necrosis virus (IHNV) encapsulated in alginate microspheres induces dose-dependent immune responses and significant protection in rainbow trout (Oncorrhynchus mykiss)

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ABSTRACT

Administered by intramuscular injection, a DNA vaccine (pIRF1A-G) containing the promoter regions upstream of the rainbow trout interferon regulatory factor 1A gene (IRF1A) driven the expression of the infectious hematopoietic necrosis virus (IHNV) glycoprotein (G) elicited protective immune responses in rainbow trout (Oncorhynchus mykiss). However, less laborious and cost-effective routes of DNA vaccine delivery are required to vaccinate large numbers of susceptible farmed fish. In this study, the pIRF1A-G vaccine was encapsulated into alginate microspheres and orally administered to rainbow trout. At 1, 3, 5, and 7 d post-vaccination, IHNV G transcripts were detected by quantitative real-time PCR in gills, spleen, kidney and intestinal tissues of vaccinated fish. This result suggested that the encapsulation of pIRF1A-G in alginate microparticles protected the DNA vaccine from degradation in the fish stomach and ensured vaccine early delivery to the hindgut, vaccine passage through the intestinal mucosa and its distribution thought internal and external organs of vaccinated fish. We also observed that the oral route required approximately 20-fold more plasmid DNA than the injection route to induce the expression of significant levels of IHNV G transcripts in kidney and spleen of vaccinated fish. Despite this limitation, increased IFN-1, TLR-7 and IgM gene expression was detected by qRT-PCR in kidney of vaccinated fish when a 10 μg dose of the oral pIRF1A-G vaccine was administered. In contrast, significant Mx-1, Vig-1, Vig-2, TLR-3 and TLR-8 gene expression was only detected when higher doses of pIRF1A-G (50 and 100 μg) were orally administered. The pIRF1A-G vaccine also induced the expression of several markers of the adaptive immune response (CD4, CD8, IgM and IgT) in kidney and spleen of immunized fish in a dose-dependent manner. When vaccinated fish were challenged by immersion with live IHNV, evidence of a dose—response effect of the oral vaccine could also be observed. Although the protective effects of the oral pIRF1A-G vaccine after a challenge with IHNV were partial, significant differences in cumulative percent mortalities among the orally vaccinated fish and the unvaccinated or empty-plasmid vaccinated fish were observed. Similar levels of protection were obtained after the intramuscular administration of 5 μg of pIRF1A-G or after the oral administration of a high dose of pIRF1A-G vaccine (100 μg); with 70 and 56 relative percent survival values, respectively. When fish were vaccinated with alginate microspheres containing high doses of the pIRF1A-G vaccine (50 or 100 μg), a significant increase in the production of anti-IHNV antibodies was detected in serum samples of the vaccinated fish compared with that in unvaccinated fish. At 10 days post—challenge, IHNV N gene expression was nearly undetectable in kidney and spleen of orally vaccinated fish which suggested that the vaccine effectively reduced the amount of virus in tissues of vaccinated fish that survived the challenge. In conclusion, our results demonstrated a significant increase in fish immune responses and resistance to an IHNV infection after the oral administration of increasing concentrations of a DNA vaccine against IHNV encapsulated into alginate microspheres.

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1. Introduction

Aquaculture is the fastest growing food-production sector in the world, providing a significant supplement to, and substitute for, wild aquatic organisms. However, viral diseases are a primary constraint to the growth of many aquaculture species. Infectious hematopoietic necrosis virus (IHNV) is, in large part, responsible for important losses in the salmonid farming industry worldwide. Economic losses are due not only to fish mortality but also to the quarantine, restriction of movement or destruction of IHNV-infected fish stocks. IHNV is a non-segmented, enveloped, single-stranded, negative-sense RNA virus belonging to the genus *Novirhabdovirus* in the family Rhabdoviridae. The genome of the virus (11 Kb) contains six open reading frames in the following order: nucleocapsid (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), non-structural viral protein (NV), and polymerase genes (L) [1,2]. Previous studies demonstrated that the IHNV G protein is the only viral protein capable of inducing a neutralizing antibody response to IHNV [3]. The virus was first detected in the Pacific Northwest in the USA and is considered endemic in this area. Despite regulatory controls to prevent its dissemination, the virus has extended its geographical reach to European and Asiatic countries, usually by the movement of infected eggs or fish. Following a disease outbreak, surviving fish may be asymptomatic carriers for life.

Preventive measures, such as effective vaccines, are critical for a sustainable development of the aquaculture industry and have been the focus of extensive research. In addition to reducing the severity of disease losses, vaccines also reduce the need for antibiotics, leave no residues in the environment and do not induce pathogen resistance. Although different types of vaccines against fish viral diseases have been described, DNA vaccines have proven particularly effective. In fact, effective DNA vaccination of a large number of fish species against a variety of viral diseases has been demonstrated [4,5]. The first demonstration that the intramuscular (i. m.) injection of a plasmid DNA encoding the IHNV G gene into fish resulted in the transient expression of the encoded gene and in the generation of protective immunity against an IHNV challenge was reported by Anderson and co-workers [6]. This DNA vaccine was designed to express the IHNV G gene under the control of the cytomegalovirus immediate early promoter (CMVIEP). Later, a DNA vaccine against IHNV containing the CMV promoter in place of the CMVIEP promoter was patented in Canada and approved for commercialization in July 2005 by the Canadian Food Inspection Agency [7]. However, the insertion of a promoter from a human pathogen (i.e. the CMV promoter), makes DNA vaccines containing this promoter "unsafe" for some countries licensing agencies. Therefore, DNA plasmid vectors containing rainbow trout specific promoters to take the place of the human CMV promoter were constructed [8]. One of these expression vectors, pIRF1A-G, contained the IHNV G gene linked to the promoter region upstream of the rainbow trout interferon regulatory factor 1A gene (IRF1A). Administered by intramuscular (i. m.) injection, the effectiveness of pIRF1A-G as IHNV DNA vaccine compared favourably with that pCMVIEP-G in vaccine trials. Although DNA vaccines against IHNV and the related fish rhabdovirus, viral hemorrhagic septicaemia virus (VHSV), have been shown to stimulate the production of specific neutralizing antibodies and to induce the expression of non-specific IFN-inducible antiviral genes such as Mx-1 and Vig-1 in rainbow trout [9,10], the specific immune mechanisms and correlates of protection of pIRF1A-G vaccinated fish remain to be elucidated.

DNA vaccination by i. m. injection is very effective in inducing fish immune responses. However, alternative routes of DNA vaccine delivery are desirable for less stressful manipulation of the fish and for use in small fish for which i. m. delivery is not practical or cost effective [11]. Oral delivery of DNA vaccines is considered the most appropriate way to immunize large numbers of small farmed fish. Advantages of oral vaccine delivery in fish include its safe, easy application, limited stress effects, and reduced cost, time and labour. However, oral vaccine delivery has some drawbacks mainly due to the strong physiological conditions encountered in the first portions of the fish gastrointestinal tract where very low pH levels may be present [12,13]. Therefore, some antigen-encapsulation methods have been developed to overcome vaccine degradation in the fish stomach and to ensure the arrival of enough quantity of plasmid vaccine to the second segment of the fish gut where uptake occurs [14,15].

We have previously conducted several studies using an oral DNA vaccine against infectious pancreatic necrosis virus (IPNV), a member of the *Birnaviridae* family that causes widespread mortality in salmonid fish. The pcDNA-VP2 vaccine, a plasmid vector encoding the VP2 gene of IPNV, was encapsulated into alginate microspheres for oral delivery to rainbow trout [16–18]. The effectiveness of the pcDNA-VP2 vaccine was demonstrated when the vaccine was orally administered by pipette directly into the mouth of both brown trout (*Salmo trutta*) and rainbow trout (*Onchorrynkuss mykiss*) or incorporated into food pellets [19]. After the oral administration of the vaccine, the VP2 transgene was expressed in several organs of vaccinated fish, induced innate and specific immune responses, and a strong protection against an IPNV challenge. At 30 days post–challenge with live IPNV, relative percent survival (RPS) values of 84 in brown trout and between 67 and 83 in rainbow trout were obtained. By using an oligo microarray, high transcriptional levels of a group of immune-related genes, including IFN-1, Mx-1, Mx-3, IgM and IgT, were detected in kidney of orally vaccinated fish prior challenge suggesting that these genes might be important for protection against IPNV [17] Overall, these studies demonstrated the potential of using alginate microspheres as an effective strategy to deliver DNA vaccines against fish viral diseases and provided some protection correlates that could be used to test efficacy of oral DNA vaccines.

In the current study, we tested whether the pIRF1A-G vaccine could also be effective when encapsulated in alginate microspheres and delivered orally to rainbow trout. The protective immune responses generated by two routes of administration of the pIRF1A-G vaccine including intramuscular injection and oral delivery in alginate microparticles were compared. For this purpose, the expression of IHNV G m-RNA transcripts and mRNA expression profiles of several markers of the innate and adaptive immune responses including IFN-1, Mx-1, Mx-3, Vig-1, Vig-2, TLR-3, TLR-7, TLR-8, CD4, CD8, IgM and IgT genes, were examined in kidney and spleen of vaccinated fish. In addition, the efficacy of the pIRF1A-G vaccine (given orally or by i. m. injection) in inducing anti-IHNV antibodies and protection against an IHNV challenge, was evaluated. Finally, we tested whether the oral administration of pIRF1A-G would effectively reduce viral load in vaccinated fish.

2. Materials and methods

2.1. Ethics statement

All the *in vivo* procedures were performed in strict accordance with the recommendations in the European Union Ethical Guidelines for the care of animals used for experimental and other scientific purposes (2010/63/EU). All the experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Consejo Superior de Investigaciones Científicas (CSIC).

2.2. Fish rearing conditions

Healthy rainbow trout specimens (3–4 ± 0.3 g mean weight) were purchased from a spring water farm with no history of viral diseases (Guadalajara, Spain). Fish were maintained under a 12-h-light/12-h-dark photoperiod at 13 ± 1 °C in 350-L closed flow-through water tanks (Living Stream, Frigid Units Inc., Ohio) at the "Centro de Investigaciones Biológicas" (CSIC, Madrid, Spain). Fish were fed daily (1.5% body weight) with a pelleted diet (Skretting, Spain). A pool of five trout was tested to confirm the absence of IHNV or any other salmonid virus by isolation using BF2 cells [20]. Prior to any experimental procedure, fish were acclimatized to laboratory conditions for 2 weeks, and during this period, no clinical signs were observed.

2.3. Encapsulation of the pIRF1A-G DNA vaccine in alginate microspheres

The pIRF1A-G vaccine, which contains the promoter regions of the rainbow trout interferon regulatory 1A gene driven the expression of the IHNV glycoprotein gene, was kindly provided by Dr. Marta Alonso and Dr. Jo-Ann Leong. The pIRF1A-G vaccine is covered by a patent application through Oregon State University (WO200269840) [21]. Construction of the pIRF1A-G vaccine was previously reported [8]. An empty plasmid lacking the IHNV G gene (pIRF1A) was used as plasmid control in the immunization experiments. Large-scale preparations of the pIRF1A-G vaccine and pIRF1A empty plasmid were prepared from lysates of *Escherichia coli* TOP10 super competent cells grown in presence of ampicillin (Life Technologies, Alcobendas, Spain). The plasmid DNAs were purified with the Endofree Plasmid Maxi purification kit according to the manufacturer's instructions (Qiagen Iberia, Spain). Purified pIRF1A-G vaccine and pIRF1A empty plasmid (1.5 mg) were encapsulated into alginate microspheres as previously described [16]. Briefly, 2.5 mL of 3% (w/v) sodium alginate were mixed with 1.5 mL of 1 mg mL^{-1} of pIRF1A-G or pIRF1A and the mixture stirred at 500 rpm during 10 min. This solution was added to an Erlenmeyer flask containing 100 mL of paraffin oil and 0.5 mL Span 80, and the mixture was emulsified for 30 min at 900 rpm. Microspheres were prepared by adding 2.5 mL of 0.15 M CaCl$_2$ to the emulsion drop by drop and stirring for 2 h at 900 rpm. Microspheres were collected by centrifugation at 1000 g for 10 min, and they were washed twice with 70% ethanol, lyophilized for 24 h and stored at 4 °C. The DNA content of the pIRF1A-G-loaded alginate microspheres was assessed by resuspending plasmid-loaded alginate microspheres in 5 mL of sodium citrate (55 mM) overnight at room temperature. The absorbance of the supernatant was measured at 260 nM in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

2.4. Immunization trials

In the first laboratory trial six experimental groups of 50 fish each (mean weight 3–4 g) were compared: (1) fish were orally immunized with a suspension of alginate microparticles containing 10 μg of pIRF1A-G, (2) fish orally immunized with 10 μg of pIRF1A-G in alginate microparticles and boosted 15 days later with the same amount of plasmid, (3) fish orally immunized with alginate microparticles containing 25 μg of pIRF1A-G, (4) fish orally vaccinated with 25 μg of pIRF1A-G in alginate microparticles and boosted 15 days later with the same amount of DNA, (5) fish orally immunized with alginate microparticles containing 10 μg of pIRF1A empty plasmid, and (6) control, unvaccinated fish. For oral immunization, fish were anaesthetised by immersion in 50 μg mL^{-1} of buffered tricaine methane sulphonate (MS-222; Sigma–Aldrich, Madrid, Spain) and then the corresponding amount of plasmid-loaded microspheres was introduced into the mouth of each fish with an automatic pipette supporting a 20 μL tip end at the entrance of the oesophagus.

In the second experimental trial, four experimental groups of fish (n = 50) were compared, including: (1) fish orally immunized with alginate microparticles containing 100 μg of pIRF1A-G, (2) fish injected at the base of the dorsal fin with 5 μg of pIRF1A-G, (3) fish injected with 5 μg of pIRF1A empty plasmid, and (4) control, unvaccinated fish.

2.5. Tissue collection, RNA extraction, cDNA synthesis and quantification of gene expression by a two-step quantitative reverse-transcription PCR (qRT-PCR)

Fish were sacrificed via MS-222 overdose. Gills, head kidney, spleen, and/or intestinal tissues were aseptically collected from vaccinated and unvaccinated trout and individually stored in 1 mL of TRIzol LS Reagent (Invitrogen, Spain) at − 70 °C until RNA isolation. Tissue samples were homogenized using the Tissue Lyser Cell Disruptor (Qiagen S. A., Madrid, Spain) 5 min at 50 Hz with 2 mm glass beads. Total RNA was isolated from different tissues by using the TRIzol LS Reagent according to the manufacturer's instructions. RNAs were treated with DNase I-RNase Free (Fermentas, Spain) to remove genomic DNA that might interfere with the PCRs. The purity and the yield of the RNA samples were analysed in a NanoDrop™ 1000 spectrophotometer. RNA quality was determined by measuring the 260/280-nm absorbance ratio, and ratios of 1.8 or higher were considered acceptable for purified RNA. Total RNA (5 μg) was then reverse transcribed to c-DNA using the Super Script™ III cDNA synthesis kit and oligo-(dT) primer (25 pmol/μL) according to the manufacturer's instructions (Life Technologies). The cDNA was diluted 1:4 in DEPC treated water and 2 μL of the diluted cDNA was used in each real-time qPCR reaction. Real-time qPCR reactions for the amplification of each target gene were performed in a 25 μL final volume containing 12.5 μL of 2 × Quantimix Easy SYBR Green (Biotools Labs, Madrid, Spain), 0.3 μM of forward and reverse primers, 8.5 μL of ultrapure water and 2 μL of diluted cDNA. Real-time qPCR amplifications of c-DNA were accomplished in an iQ5 iCycler Real-Time PCR Detection System (Bio-Rad Laboratories, Madrid, Spain) under the following conditions: 1 cycle of 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min, and a dissociation cycle (1 min at 95 °C and 1 min at 60 °C). After the run, the melting curve of each amplicon was analysed to determine the specificity of the amplification. Table 1 shows the list of the amplified trout genes, their accession numbers and corresponding primer sequences. Since the trout Elongation Factor 1α (EF-1α) is constitutively expressed, it was used as the endogenous gene control to normalize the expression of each target gene in each RNA sample. PCR amplifications of the IHNV G gene were carried out using TaqMan probes and specific primers as previously described [16]. The rainbow trout β-actin gene was used as the endogenous control gene for the normalization of the IHNV G gene expression. Results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as $2^{-\Delta CT}$ where ΔC_T is determined by subtracting the EF-1α (or actin) threshold cycle (C_T) from the target C_T value [22]. Negative controls with no template where included in all experiments. All the PCR amplifications were performed by duplicate, and each experiment was repeated twice to confirm the results.

2.6. IHNV experimental challenge by immersion

At 30 days post-vaccination (p.v.), duplicate sets of 15 fish (average weight of 4 g) from each treatment group were challenged

Table 1
Genes and primer sequences used in the qRT-PCR assays.

Code	Name	Abbreviation	Primer code/sequence (5′-3)
NM_001124531	Type I Interferon	IFN-1	F/AAAACTGTTTGATGGGAATATGAAA R/CGTTTCAGTCTCCTCTCAGGTT
NM_001171901	Interferon-induced protein Mx1	Mx-1	F/AGCTCAAACGCCTGATGAAG R/ACCCCACTGAAACACACCTG
U47946.1	Interferon-induced protein Mx3	Mx-3	F/AGCTCAAACGCCTGATGAAG R/TGAATATGTCTGTTATCCTCCCAAA
AF076620.1	Viperin 1	Vig-1	F/ACGACCTCCAGCTCCCAAGT R/GTCCAGGTGGCTCTTCCTGC
AF290477.1	Viperin 2	Vig-2	F/CCACCCACGTCATATCAGGG R/AACGCAGACGCCTTGTTGGC
ABE69177	Toll-like receptor 3-like protein	TLR-3	F/AGCCCTTTGCTGCCTTACAGAG R/GTCTTCAGGTCATTTTTGGACACG
GQ422119	Toll-like receptor 7	TLR-7	F/TACAGCTTGGTAACATGACTCTCC R/CAACTCTCTGAGACTTGTCGGTAA
GQ422120	Toll-like receptor 8α2	TL8α2	F/CAGCATTGAACGGGACAGAG R/CGTTGTCATAGGCCAGGTCA
AY973030.1	T-cell surface glycoprotein CD4	CD4	F/CCTGCTCATCCACAGCCTAT R/CTTCTCCTGGCTGTCTGACC
NM_001124263	T-cell surface glycoprotein CD8 alpha precursor	CD8	F/AGTCGTGCAAAGTGGGAAAG R/GGTTGCAATGGCATACAGTC
X65263.1	Membrane bound Immunoglobulin M	IgM	F/ACCTTAACCAGCCGAAAAGGG R/TGTCCCATTGCTCCAGTCC
AY870265	Immonoglobulon Tau heavy chain	IgT	F/AGCACCAGGGTGAAACCA R/GCGGTGGGTTCAGAGTCA
L40883	Nucleocapsid protein	N	F/TGTGCATGAAGTCAGTGGTGG R/CCTGCTCATCATGACACCGTA
AF498320	Elongation factor EF1 alpha	EF-1α	F/GATCCAGAAGGAGGTCACCA R/TTACGTTCGACCTTCCATCC
Y18854.1	Glycoprotein protein	G gene	F/GCGCACGCCGAGATAATATCAA R/TCCCGTGATAGATGGAGCCTTT P/CGATCTCCACATCCCGGAATAAATGACGTCT
AJ438158	Actin beta	β-actin	F/GGCCGTGTTGTCCCTGTAC R/CCGGAGTCCATGACGATACC P/CCTCTGGCCGTACCACC

by immersion with 10^5 TCID$_{50}$ mL^{-1} of live IHNV (ATCC VR 714) for 3 h at $14 \pm 1\ ^\circ$C with aeration. Dead fish were collected daily for 30 days post−challenge. Moribund fish displayed classical signs of IHNV infection such as distended abdomens, darkened body coloration and vertical drifting. Approximately, 10% of the dead fish were assessed by PCR amplification of the IHNV N gene to confirm that they died as a result of an IHNV infection. Vaccine efficacy was assessed by comparing the final cumulative percent mortality (CPM) among vaccinated and unvaccinated fish. The relative percent survival (RPS) value of each experimental group was calculated according to the following formula: RPS = [1 − (CPM vaccinated fish/CPM unvaccinated fish)] × 100 [23].

2.7. Indirect enzyme-linked immunosorbent assay (ELISA) for the detection of anti-IHNV serum antibodies

At 15, 30 and 45 days p. v., rainbow trout were sacrificed by immersion in an overdose solution of MS-222 (200 μg mL^{-1}), and blood was collected from the caudal vein of vaccinated and un-vaccinated fish using a 25-gauge needle (Becton, Dickinson and Company, Sparks, MD, US). Blood samples were allowed to clot at room temperature for 2 h, stored at 4 $^\circ$C overnight, and then centrifuged at 500 g for 10 min. Serum samples were collected and stored at −20 $^\circ$C until they were analysed by ELISA. Briefly, 96-well microtiter plates (Greiner Bio-one, Frickenhausen, Germany) were coated with 100 μL of IHNV previously propagated in BF-2 cells; the cell culture medium was used as negative antigen control. After incubation for 18 h at 4 $^\circ$C, unbound virus was removed by washing each well five times with 200 μL of PBS with 0.05% Tween 20 (PBS-T). The plates were then blocked for 2 h at 22 $^\circ$C with blocking

solution consisting of 5% skimmed milk in PBS. The plates were washed three times with PBS-T, and 100 μL of each fish serum sample serially diluted in PBS with 1% bovine serum albumin (BSA) was added in triplicate. After 3 h of incubation at 22 $^\circ$C, the plates were washed three times with 200 μL of PBS-T. Each well was then inoculated with 100 μL of the anti-rainbow trout IgM monoclonal antibody (Aquatic Diagnostics Ltd, Stirling, Scotland) and incubated at room temperature for 1 h. Unbound antibody was removed after three washes with 200 μL of PBS-T. Each well was then inoculated with 100 μL of horseradish peroxidase-conjugated goat anti-mouse IgM polyclonal antibody (Dako, Glostrup, Denmark), diluted 1:1000 in PBS with 1% BSA and incubated at 22 $^\circ$C for 1 h. Unbound con-jugate was removed after 5 washes with 200 μL of PBS-T, and then antibody binding was visualized by adding 100 μL of 3, 3′, 5, 5′-tetramethylbenzidine dihydrochloride (TMB) to each well. The plates were incubated for 10 min at 22 $^\circ$C in the dark, and the re-actions were stopped by adding 50 μL of H_2O_2 per well. The optical density (OD) in each well was measured at 450 nm by an ELISA microplate reader (Model M680, Bio-Rad, Spain). The specific antibody titre of each sample was expressed as mean OD values (±SE) after subtracting OD values from the unvaccinated fish.

2.8. Statistical analysis

Differences in CPMs between vaccinated and control fish groups were statistically analysed, using a Fisher's exact test (GraphPad Prism version 4.03, GraphPad Software, San Diego, CA). Differences in gene expression between experimental groups were analysed using factorial ANOVAs with the Tukey−Kramer adjustment for multiple comparisons (IBM SPSS Statistics 15; Chicago, IL, US). A

common unvaccinated control group was used to determine the statistical significance of the gene expression levels obtained from all the immunized experimental groups. In all analyses, differences between groups were considered statistically significant when the correlation value P *was* <0.05.
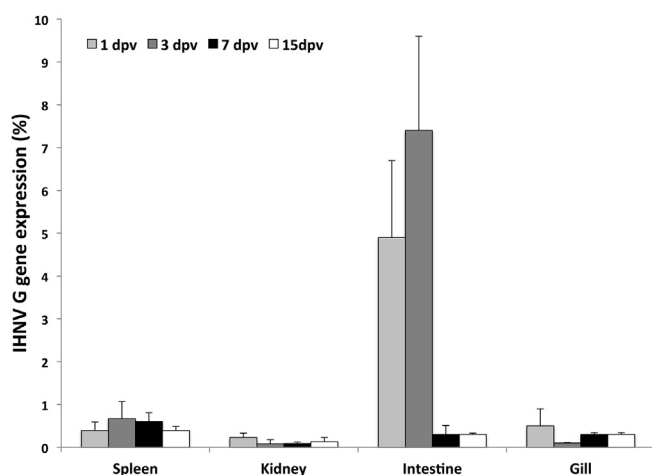
3. Results

3.1. IHNV G transcripts were expressed in several fish organs after oral vaccination with pIRF1A-G encapsulated in alginate microspheres

To verify whether alginate microspheres protected the pIRF1A-G vaccine from stomach degradation, IHNV G expression was examined in gills, kidney, spleen, and intestinal tissues of rainbow trout vaccinated with a 10 μg dose of the vaccine. At 1, 3, 7 and 15 days p. v., low but appreciable levels of transgene expression were observed in all the examined organs (Fig. 1). When the G gene expression in the four examined organs was compared, the lowest expression levels were detected in kidney of vaccinated fish at the four assessed time points. In contrast, the highest IHNV G gene expression levels were recorded in intestinal tissues of the vaccinated fish at 1 and 3 days p. v. These findings suggested that the encapsulation of pIRF1A-G vaccine in alginate microspheres ensured the early delivery of the vaccine to the hindgut, its passage through the intestinal mucosa and its distribution thought internal and external organs of vaccinated fish.

3.2. Expression of IHNV-G, TLRs, IFN-1, and IFN-inducible genes in response to the oral or intramuscular administration of the pIRF1A-G vaccine

We compared the transcriptional profiles of several host immune markers after the oral pIRF1A-G immunization with those induced after pIRF1A-G injection. Some genes previously found highly expressed (>2-fold) in oral pcDNA-VP2 vaccinated trout and/or induced after IPNV-infection were selected for the current

analysis [18]. The selected genes belonged to (i) the innate immune response such as IFN-1 [24,25], Mx-1 [26], Mx-3 [27], Vig-1 [28], Vig-2 [29], TLR-3 [30], TLR-7, TLR-8 [31]; and (ii) the adaptive immune response such as IgM [32,33], IgT [34], CD4 and CD8 [35]. Table 1 shows the corresponding primer sequences designed for the qRT-PCR analysis of those genes. Because head kidney and spleen are both target organs of IHNV multiplication and two of the most important trout immune-responsive organs, they were both selected as target organs for transcriptional analysis. In order to determine whether increasing doses of the oral pIRF1A-G vaccine would significantly increase the expression of the selected immune markers, groups of rainbow trout were immunized with alginate microparticles containing 10, 25 or 100 μg of pIRF1A-G-loaded alginate microparticles. Fish intramuscularly injected with 5 μg of pIR1A-G and unvaccinated fish were used as positive and negative controls, respectively. At 3, 7 and 15 days p. v., kidney and spleen were collected from four fish of each group and analysed for gene expression by qRT-PCR. The transcription levels of the rainbow trout Mx-1, Mx-3, Vig 1, Vig 2, TLR-3, TLR-7 and TLR-8 genes in kidney and spleen of vaccinated and unvaccinated animals were normalised to the expression of the endogenous control, Elongation factor 1α (EF-1α), and the corresponding results are shown in Figs. 2 and 3 according to the assessed organ. The expression of the IHNV G gene was also included in the analysis as a vaccine marker. As expected, our results demonstrated that the expression of the IHNV G transgene in kidney and spleen of vaccinated animals increased with increasing concentrations of the oral vaccine. Since we previously observed that empty plasmids only induced marginal increases in host gene expression and our goal was to compare protective immune responses induced by two routes of vaccine delivery, a common unvaccinated control group was used to determine the statistical significance of the gene expression levels obtained from all the immunized experimental groups.

Of the three assessed doses of oral vaccine, only the 100 μg dose induced significant levels of IHNV G transcripts in kidney and spleen of vaccinated fish. Furthermore, higher levels of IHNV G expression were detected in kidney of fish orally vaccinated with 100 μg of pIRF1A-G vaccine than in injected fish at all the assessed time points. Only at day 7 p. v., higher levels of IHNV G expression were detected in spleen of fish orally vaccinated with the 100 μg dose when compared with the injected fish.

*G*ene expression analysis in *kidney of oral and intramuscular vaccinated fish showed* significant increases in the expression of most of the examined host genes (Fig. 2). After oral vaccination increased IFN-1 and TLR-7 gene expression was observed in kidney of vaccinated fish even when the lowest dose of the vaccine was administered. Statistical analysis of the date indicated that the Mx-1, Vig-2, and TLR-3 genes were highly expressed in fish orally vaccinated with a vaccine dose ≥25 μg. A significant increase in Vig-1, Mx-3 and TLR-8 gene expression was only detected in kidneys of the fish orally vaccinated with the highest dose of the DNA vaccine (100 μg). Remarkably, a significant induction of IFN-1 gene expression was detected after immunization with the three assessed doses of oral DNA vaccine and at 3, 7 and 15 days p.v. When we compared the two routes of vaccine delivery, we detected similar IFN-1 gene expression in kidney of injected fish and in the fish groups vaccinated with the oral DNA vaccine. However, significant differences in the expression of the Mx-1 at day 3 p. v., Mx-3 at day 7 p. v. and TLR-7 and TLR-8 genes at days 7 and 15 p. v. were observed depending on the vaccine delivery method. Interestingly, the administration of 100 μg of the oral vaccine induced high levels of TLR-7 and TLR-8 gene expression over time, which at 7 and 15 days p. v. were higher than the levels induced by the intramuscular injection of the DNA vaccine, while no differences were recorded in the first 3 days p. v. As seen in Fig. 2, the increase in TLR-7 and TLR-8
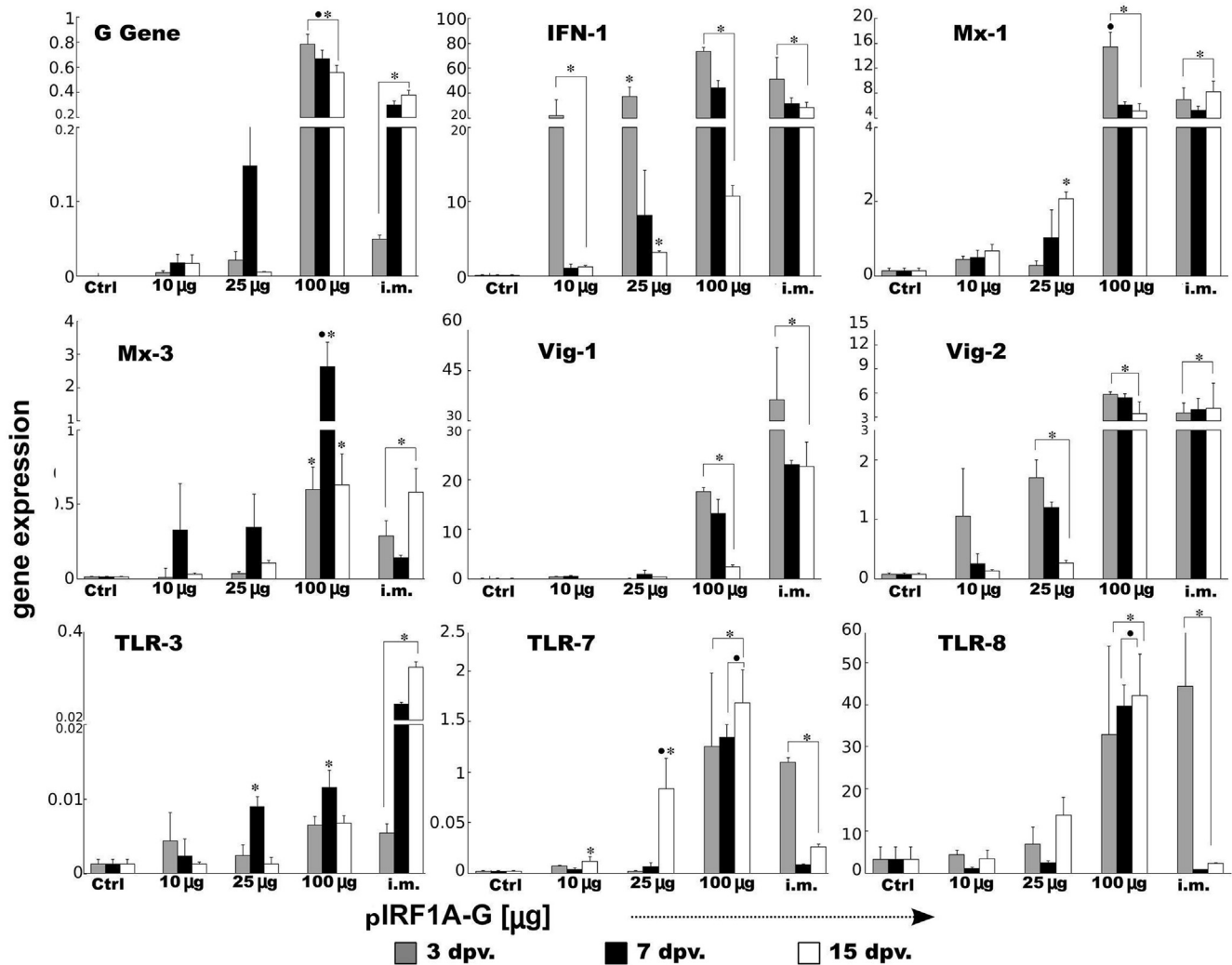

Fig. 1. IHNV G gene expression in trout tissues after oral immunization with pIRF1A-G-loaded alginate microparticles. Rainbow trout were immunized with 10 μg of alginate-encapsulated pIRF1A-G vaccine. IHNV G expression was analysed by qRT-PCR in gills, kidney, spleen and intestinal tissues collected from four vaccinated rainbow trout 1, 3, 7 and 15 days after immunization. Results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as the mean $2^{-\Delta C_T}$ where ΔC_T is the Ct (target gene) − Ct (EF-1α). In the figure, IHNV G gene expression is presented as a percentage of the initial amount of vaccine orally given (10 μg).
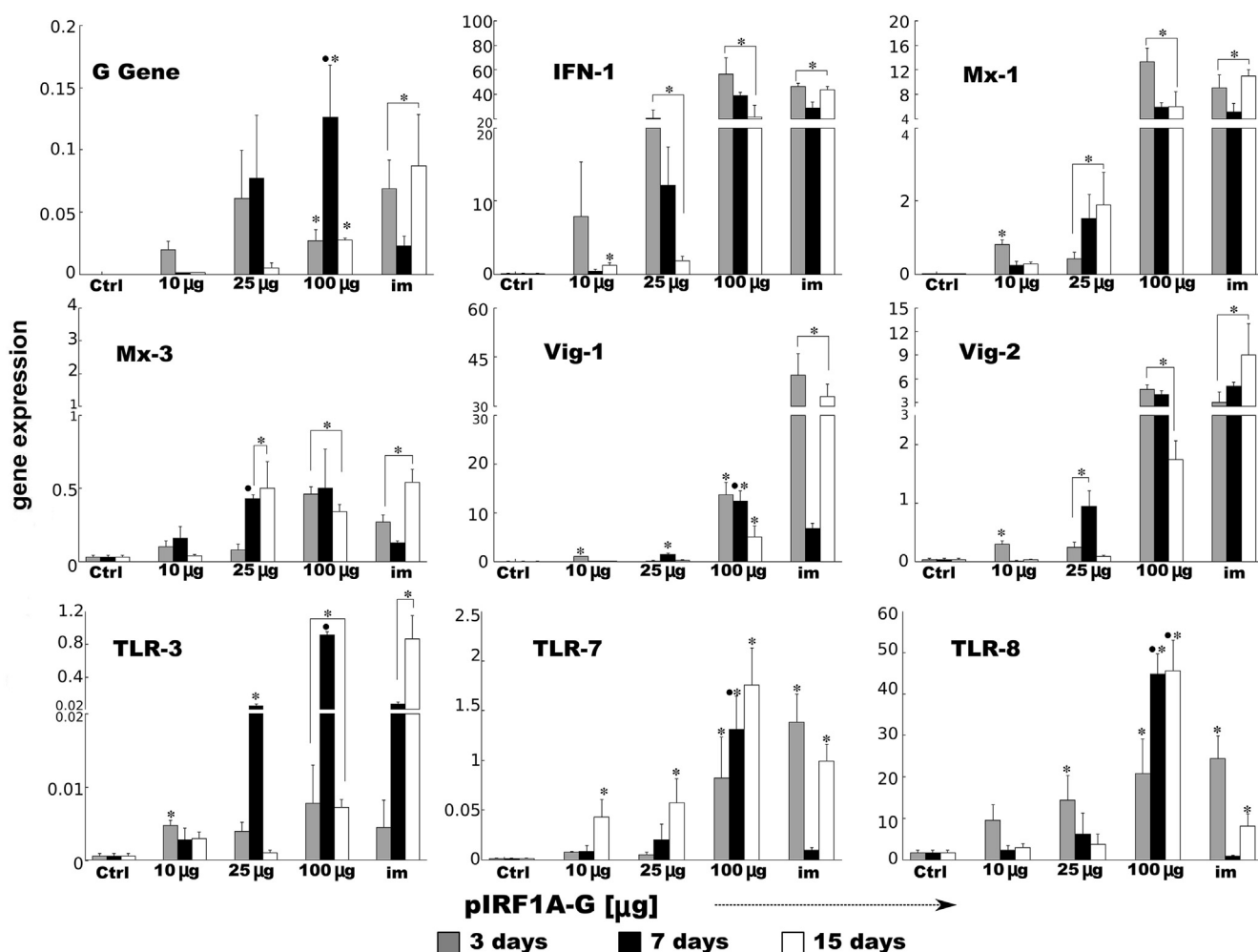
Fig. 2. IHNV-G, TLRs, IFN-1, and IFN-stimulated gene expression in kidney after oral immunization of rainbow trout with increasing concentrations of pIRF1A-G-loaded alginate microparticles. Rainbow trout were immunized with 10, 25 and 100 μg of alginate-encapsulated pIRF1A-G vaccine. IFN-1, Mx-1, Mx-3, Vig-1, Vig-2, TLR-3, TLR-7, and TLR-8 gene expression was evaluated by qRT-PCR in kidney of four vaccinated fish at 1, 3, 7 and 15 d p. v. The analysis of the IHNV G expression was also included as a vaccine marker. Fish intramuscularly injected with 5 μg of pIR1A-G and unvaccinated fish were used as positive and negative controls, respectively. qRT-PCR results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as $2^{-\Delta CT}$ where ΔC_T is the mean Ct (target gene) − Ct (EF-1α). Asterisks indicate significant differences of expression ($P \leq 0.05$); black points indicate significant differences between oral and intramuscular vaccinated fish.

gene expression 3 days after the intramuscular administration of the vaccine was an initial response which was then quickly suppressed.

The transcription levels of the rainbow trout Mx-1, Mx-3, Vig 1, Vig 2, TLR-3, TLR-7 and TLR-8 genes were also examined in the spleens of the fish orally vaccinated with increasing concentrations of the oral pIRF1A-G vaccine. The corresponding results are presented in Fig. 3. As expected, a dose-dependent effect of the vaccine on gene expression was evident. When gene expression in spleen of fish injected with 5 μg of pIRF1A-G and orally vaccinated with 100 μg of the vaccine was compared, similar levels of IFN-1, Vig-2 and Mx-1 expression were detected at three assessed time points. Remarkably, the administration of 100 μg of the oral vaccine induced higher levels of TLR-3, TLR-7 and TLR-8 gene expression in spleen of the vaccinated animals at day 7 p. v. than did the intramuscular injection of the DNA vaccine. At 15 days p. v., only the TLR-8 gene remained highly expressed in spleen of orally vaccinated fish when compared with the levels of expression of this gene in i. m. injected fish.

3.3. CD4, CD8, IgM and IgT gene expression in response to the oral or intramuscular administration of the pIRF1A-G vaccine

Cellular-specific immune responses induced after the oral or intramuscular administration of pIRF1A-G vaccine were evaluated at 15 days p. v. in spleen and kidney of vaccinated fish by qRT-PCR. Four markers of the cellular immune response including the rainbow trout CD4, CD8, IgM and IgT genes were selected for gene expression analysis. As seen in Fig. 4, CD4 and CD8 expression levels were significantly higher in kidney of orally vaccinated fish (100 μg dose) than in i. m. injected fish. A lower dose of the oral vaccine (25 μg) was also able to induce significant levels of CD4 gene expression in spleen of vaccinated fish. CD8 expression levels were lower than those of CD4 regardless of the treatment and were higher in the fish vaccinated with 100 μg of pIRF1A-G than in any other experimental group. Interestingly, the i. m. injection of the vaccine did not induce significant levels of CD8 gene expression in kidney and spleen of vaccinated fish. The transcription levels of IgM and IgT genes at 15 days p. v. were also analysed by qRT-PCR in

Fig. 3. IHNV-G, TLRs, IFN-1, and IFN-stimulated gene expression in trout spleen after immunization with increasing concentrations of pIRF1A-G-loaded alginate microparticles. Rainbow trout were immunized with 10, 25 and 100 μg of pIRF1A-G-loaded alginate microparticles. IFN-1, Mx-1, Mx-3, Vig-1, Vig-2, TLR-3, TLR-7, and TLR-8 gene expression was evaluated at 1, 3, 7 and 15 d p. v. by qRT-PCR in spleen of four vaccinated fish. The analysis of the expression of the IHNV G gene was also included as a vaccine marker. Fish intramuscularly injected with 5 μg of pIR1A-G and unvaccinated fish were used as positive and negative controls, respectively. qRT-PCR results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as $2^{-\Delta CT}$ where ΔC_T is the mean Ct (target gene) − Ct (EF-1α). Asterisks indicate significant differences ($P \leq 0.05$) of expression; black points indicate significant differences between oral and intramuscular vaccinated fish.

spleen and kidney of intramuscularly and orally vaccinated fish (Fig. 4). No significant differences in IgM gene expression were observed between the injected and orally vaccinated fish regardless of the assessed tissue. Administration of the vaccine orally or by injection induced significant IgM expression in kidney tissues. Even at the lowest dose of the oral vaccine, a significant increase in IgM expression was detected in the kidney of vaccinated animals. In contrast, only the highest dose of the oral vaccine induced significant IgT expression in kidney and spleen of vaccinated animals. Remarkably, no significant production of IgT transcripts were detected in kidney of the i. m. injected fish.

3.4. Alginate-encapsulated pIRF1A-G vaccine protected orally vaccinated fish against a lethal challenge with IHNV

In the first experimental trial, increasing concentration of alginate-encapsulated pIRF1A-G vaccine were orally administered to rainbow trout fry and evaluated for their efficacy in inducing a protective immune response against an IHNV challenge. Groups of 50 rainbow trout were orally immunized with alginate microparticles containing (1) 10 μg of pIRF1A-G, (2) 10 μg of pIRF1A-G and boosted 15 days later with the same amount of plasmid, (3) 25 μg of

pIRF1A-G, and (4) 25 μg of pIRF1A-G and boosted 15 days later with the same amount of DNA. Fish orally immunized with alginate microparticles containing 10 μg of empty-plasmid and unvaccinated fish were used as negative controls. At 30 days p. v., two subgroups of 15 fish form each group were challenged by immersion with 10^5 TCID$_{50}$ mL^{-1} of IHNV. Mortality was recorded daily for 30 days. The effects of increasing concentrations of the oral DNA vaccine on CPM are shown in Fig. 5A. In general, our results showed that alginate-encapsulated pIRF1A-G vaccinated fish exhibited less mortality than the empty-plasmid vaccinated or unvaccinated fish. At 30 days post−challenge, immunization with 10, 20, 25 and 50 μg of the oral DNA vaccine resulted in CMPs of 78, 70, 70, and 54%, respectively. In contrast, the control fish that received orally the empty-plasmid and the unvaccinated animals exhibited CMPs of 90 and 100%, respectively. Although the protective effects of the oral pIRF1A-G vaccine after challenge with live IHNV were partial, statistically significant differences in mortalities between the fish orally vaccinated with 10, 20, 25 and 50 μg of the DNA vaccine and the empty-plasmid vaccinated or unvaccinated fish were observed; RPSs of 21, 30, 30 and 45, respectively. No significant differences in mortalities were observed between the empty-plasmid vaccinated fish and the unvaccinated fish ($P = 0.0015$). CMP in the challenged

pIRF1A-G (50 μg) group was 54% and 100% in the unvaccinated group, resulting in an RPS of 45.

In the second experimental trial, the effect of a higher dose of the oral pIRF1A-G vaccine (100 μg) on fish CPM was evaluated. Fish injected with 5 μg of pIRF1A-G or pIRF1A empty plasmid were used as positive and negative control groups, respectively. Unvaccinated fish were used as the negative control group for RPS calculations. As shown in Fig. 5B, immunization with 100 μg of the oral pIRF1A-G vaccine resulted in a CPM of 43.75 while the CPM in the injected group was 30% ($P = 0.0566$). In contrast, 100% and 90% of the un-vaccinated and empty-plasmid vaccinated fish succumbed to virus challenge, respectively. Therefore, both injected fish and orally vaccinated fish were significantly protected against the challenge with IHNV when compared with unvaccinated or empty-plasmid injected fish ($P \leq 0.001$). RPS values were calculated for each challenged experimental group and are shown in Fig. 5B. In sum-mary, our data indicated that the oral administration of a 100 μg dose of pIRF1A-G induced significant protection similar to that obtained when fish were vaccinated by i. m. injection with 5 μg of the DNA vaccine; 56 and 70 RPS, respectively.

3.5. Assessment of IHNV viral load in vaccinated and unvaccinated trout after challenge with live IHNV

To determine whether the oral administration of pIRF1A-G (10 μg dose) would reduce viral load in vaccinated fish, the tran-scriptional levels of the IHNV nucleoprotein gene were quantified by qRT-PCR in spleen, kidney, gills and intestinal tissues of vaccinated and unvaccinated fish that survived the IHNV challenge (Fig. 6). At

10 days post–challenge, the levels of IHNV N gene expression in vaccinated fish were lower than that quantified in unvaccinated fish regardless of the assessed organ. Furthermore, N transcript expression was highest in spleen of unvaccinated fish than in other tested organs. Conversely, N gene expression was nearly undetect-able in kidney and spleen of vaccinated fish which suggested that the oral vaccine significantly reduced the amount of virus in fish internal organs. As a consequence, the risk that vaccinated carriers will spread the disease is much lower than unvaccinated carriers.

3.6. IFN-1, Mx-1, Mx-3, Vig-1 and Vig-3 gene expression in vaccinated and unvaccinated trout after challenge with live IHNV

Ten days after challenge with IHNV, the levels of IFN-1, Mx-1, Mx-3, Vig-1 and Vig-3 gene expression were examined in kidney and spleen of three orally vaccinated fish (10 μg dose of pIRF1A-G) and the results are presented in Fig. 6B and C, respectively. Although no significant differences in the expression of Mx-1, Mx-3, Vig-1 and Vig-3 genes in kidney of vaccinated and unvaccinated fish was evident a significant increase in IFN-1 gene expression was observed 10 days after challenge in spleen of vaccinated fish when compared with unvaccinated fish.
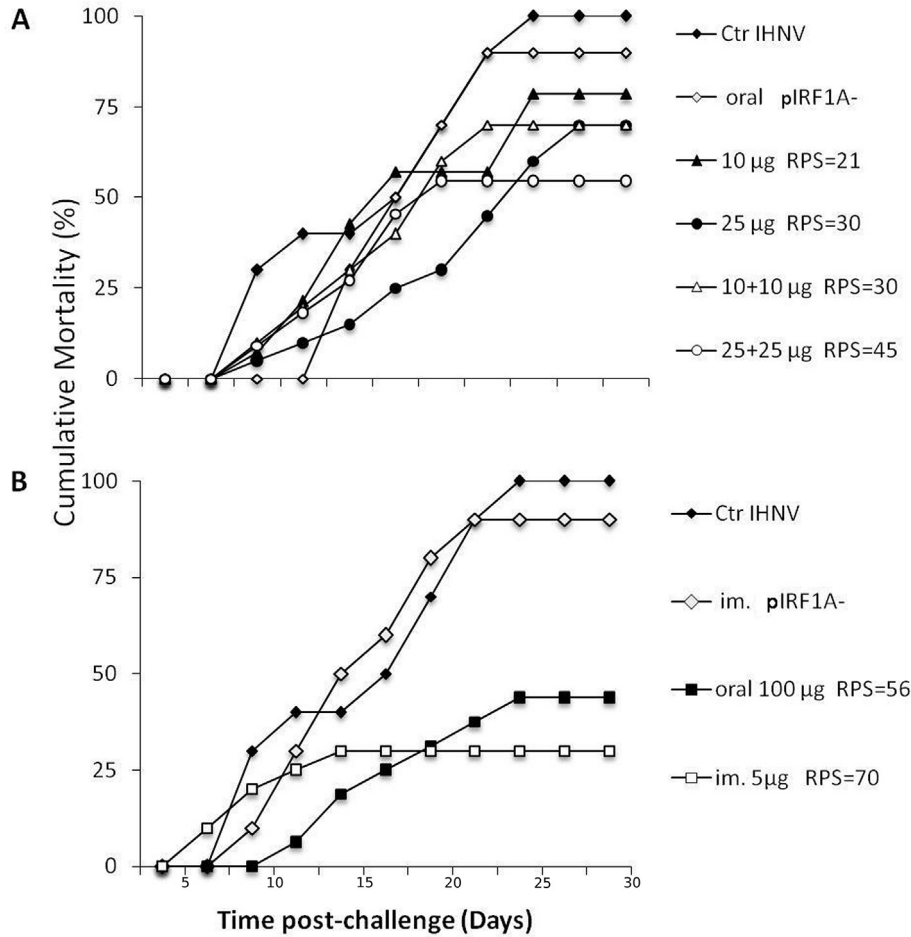
3.7. Alginate-encapsulated pIRF1A-G vaccine induced the production of anti-IHNV antibodies in the serum of the vaccinated fish

At 15, 30 and 45 days p. v. and before challenge with IHNV, blood samples were collected from the caudal vein of orally vaccinated
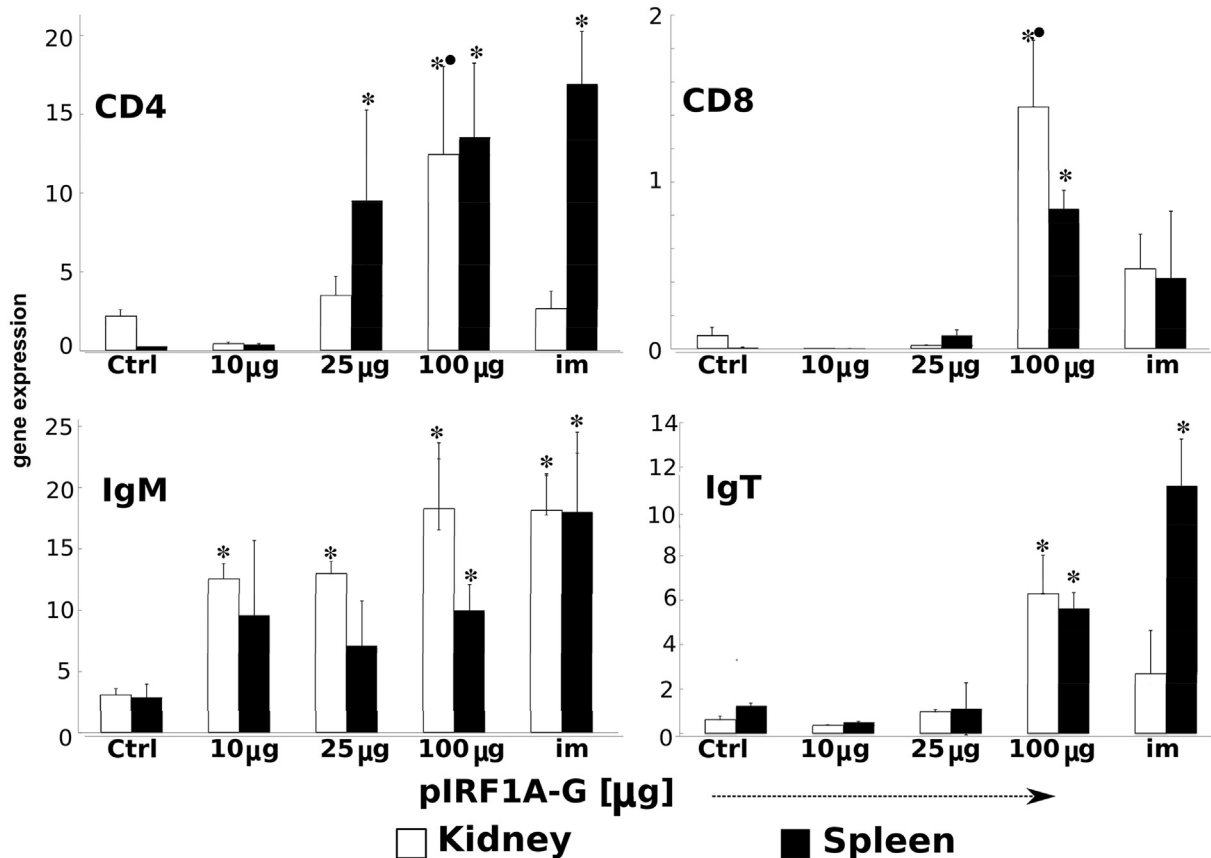

Fig. 4. CD4, CD8, IgM and IgT gene expression in trout kidneys and spleens after oral immunized with pIRF1A-G-loaded alginate microparticles. Rainbow trout were immunized with 10, 25 and 100 μg of pIRF1A-G-loaded alginate microparticles. Fish intramuscularly injected with 5 μg of pIR1A-G and unvaccinated fish were used as positive and negative controls, respectively. Gene expression was evaluated at 15 d p. v. as described in the legend of Fig. 3. Asterisks and black points indicate significant differences ($P \leq 0.05$) between vaccinated and unvaccinated fish or between oral and intramuscular vaccinated fish, respectively.

Fig. 5. A- Cumulative percent mortalities (CPM) of rainbow trout after the oral administration of the alginate-encapsulated pIRF1A-G vaccine and subsequent challenge with IHNV. A- Groups of 50 rainbow trout were orally immunized with alginate microparticles containing 10 μg of pIRF1A-G, 10 μg of pIRF1A-G and boosted 15 days later with the same amount of plasmid, 25 μg of pIRF1A-G, and 25 μg of pIRF1A-G and boosted 15 days later with the same amount of DNA. Fish orally immunized with alginate microparticles containing 10 μg of empty-plasmid and unvaccinated fish were used as negative controls. At 30 days p. v., two subgroups of 15 fish form each group were challenged by immersion with IHNV at a concentration of 10^5 $TCID_{50}$ mL^{-1}. Mortality was recorded daily for 30 days. B- In a second trial, groups of 50 trout each were immunised by i. m. injection with 5 μg of pIRF1A-G or orally with 100 μg of pIRF1A-G-loaded alginate microparticles. As controls, groups of fish were injected with 5 μg of the empty-plasmid or remained unvaccinated. After 30 days, the fish were challenged with IHNV (10^5 $TCID_{50}$ mL^{-1}) by immersion and monitored for the next 30 days. CPM of each treatment group was recorded, and the relative percent survival (RPS) was calculated using the formula RPS = [1 − (% mortality vaccinated fish/% mortality unvaccinated fish) − 100]).
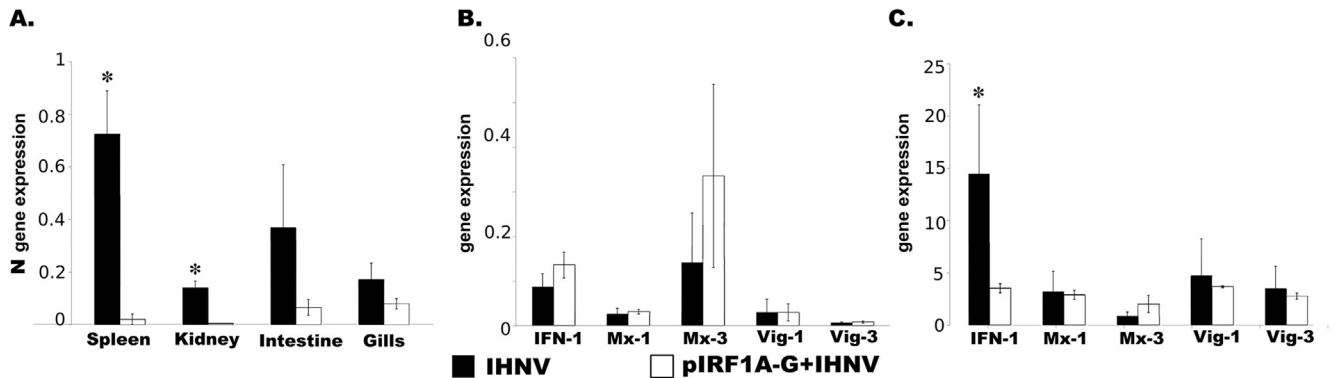
Fig. 6. Viral load (A) and cytokine gene expression (B and C) was determined in tissues of trout orally vaccinated and then challenged with IHNV. Fish orally vaccinated with 10 μg of alginate-encapsulated pIRF1A-G and unvaccinated fish were included in this assay. At 10 days post−challenge, three trout from each group were sacrificed and the RNA was individually extracted. A. The levels of expression of the IHNV N gene in spleen, kidney, intestine, and gills of unvaccinated and orally vaccinated fish were determined using qRT-PCR. B and C. IFN-1, Mx-1, Mx-3, Vig-1, and Vig-3 were assessed by qRT-PCR in kidney (B) and spleen (C) of unvaccinated and orally vaccinated fish. All qRT-PCR results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as $2^{-\Delta CT}$ where ΔC_T is the mean Ct (target gene) − Ct (EF-1α). Values with asterisks are statistically different ($P \leq 0.05$).

fish (50 or 100 μg doses of pIRF1A-G), and from fish i. m. injected with 5 μg of pIRF1A-G (n = 10). The corresponding serum samples were tested for anti-IHNV antibodies by ELISA. As seen in Fig. 7, anti-IHNV antibodies were produced in response to the oral and intramuscular administration of pIRF1A-G. Remarkably, no significant differences in antibody production were detected between the three groups of vaccinated fish at the three assessed p. v. time points.

4. Discussion

A DNA vaccine (pIRF1A-G) containing the promoter regions upstream of the rainbow trout IRF1A gene driven the expression of the IHNV glycoprotein previously conferred a high level of protection against an IHNV challenge when administered by intramuscular injection [8]. Although effective, injection route is labour intensive and only practiced for high-value species. Alternatively, the current study represents the first description of successful oral immunization of rainbow trout against a fish *Novirhabdovirus*.

Following oral immunization with 10 μg of pIRF1A-G-loaded alginate microparticles, we were able to detect IHNV G transgene expression by qRT-PCR in gills, spleen, kidney, and intestinal tissues of vaccinated fish. The highest IHNV G gene expression levels were recorded in intestinal tissues early after the oral administration of pIRF1A-G (1 and 3 d p. v.), suggesting that alginate microparticles effectively protected the DNA vaccine avoiding its degradation in the fish stomach at least to some degree. From our results, we can also infer that the oral pIRF1A-G vaccine crossed the intestinal epithelium by a still unknown mechanism, was transported into the blood and was able to reach several internal and external organs of the fish. Consequently, IHNV-G transcripts could be detected in spleen, kidney and gills of vaccinated fish at 1, 3, 7 and 15 days after vaccination. When G gene expression in the four examined organs was compared, the lowest expression levels were detected in kidney of vaccinated fish at the four assessed time

points. As we previously suggested, the fish head kidney might be acting as a scavenger tissue, clearing the blood from circulating plasmid DNA [18].

As expected, our results demonstrated that the expression of IHNV G transcripts in kidney and spleen of vaccinated trout increased when increasing concentrations of pIRF1A-G loaded alginate microparticles. Although alginate microparticles are very resistant to acidic pH, which impedes degradation of the vaccine in the stomach of the fish (pH 2–4) and favours its release in the foregut and hindgut (pH 7.0 and 8.3, respectively), our results indicated that the oral route required approximately 20-fold more plasmid DNA than the injection route to achieve significant levels of IHNV G expression in fish internal organs. After the vaccine pass through the intestinal epithelium, however, similar IFN-1 and Vig 2 gene expression was detected in kidney and spleen of vaccinated fish at 3, 7 and 15 days p. v., regardless of the administration route. Moreover, a low dose of the oral vaccine (10 μg) was able to stimulate the expression of IFN-1, TLR-7 and IgM genes in kidney of orally vaccinated fish. In addition, the oral administration of 10 μg of pIRF1A-G vaccine resulted in a significant induction of Mx-1, Vig-1, Vig-2 and TL-7 gene expression at 3 d p. v. in spleen of vaccinated trout. Remarkably, IFN-1 gene expression was significantly induced by the three tested doses of the oral DNA vaccine (10, 25 and 100 μg) in spleen and kidney of vaccinated fish. In contrast, significant Mx-1, Vig-1, Vig-2, TLR-3 and TLR-8 gene expression was only detected when high doses of pIRF1A-G vaccine were orally administered (50 and 100 μg).

TLRs are transmembrane proteins that recognise conserved pathogen structures. In fish, several studies have reported that TLRs expression is regulated by viral infections [31,36]. Our results suggested that TLR-3, TLR-7 and TLR-8 expression was affected by the oral and intramuscular administration of pIRF1A-G vaccine. Therefore, these TLRs might be acting as important mediators of IHNV DNA vaccines in rainbow trout. Remarkably, the administration of 100 μg of the oral vaccine induced higher levels of TLR-3, TLR-7 and TLR-8 gene expression in spleen of the vaccinated animals at day 7 p. v. than did the intramuscular injection of the DNA vaccine. It remains to be elucidated whether oral IHNV DNA vaccination with pIRF1A-G-loaded alginate microparticles mimics several other transcriptional responses occurring after infection with IHNV. Previously, we demonstrated that the oral-alginate VP2-vaccination immunizes trout against IPNV in a similar way as IPNV infection does [18].

Although similar levels of IFN-1 and Vig-2 gene expression were detected in kidney and spleen of vaccinated fish regardless of the administration route, several studies have shown that the intramuscular administration of a vaccine stimulates a different immune response from that stimulated by administration of antigens orally [37–39]. In fish, as in higher vertebrates, there is a very complex mucosa associated lymphoid tissue (MALT) in the gut (GALT), skin (SALT), nasopharynx (NALT) and gills (GIALT), with a specific and specialized immune response for these tissues [14,39,40]. In the fish intestine, for instance, CD8+ T cells dominate the CD4+ subset and the number of such cells increases from the foregut to the hindgut [41]. Oral and anal administration in fish activates GALT, while injection generally does not activate this stimulatory route [14]. Although we did not study local GALT immune responses, which might be very important after oral vaccination, CD4 (a classical marker of T helper cells) and CD8 (a marker of cytotoxic lymphocytes) transcripts were measured in response to the oral or intramuscular administration of pIRF1A-G vaccine in internal organs of the vaccinated fish at 15 days p. v. Our results showed higher CD4 and CD8 expression levels in kidney of fish vaccinated with 100 μg of the oral vaccine than in the i.m. injected fish. Intramuscularly injected fish did not show a significant increase in
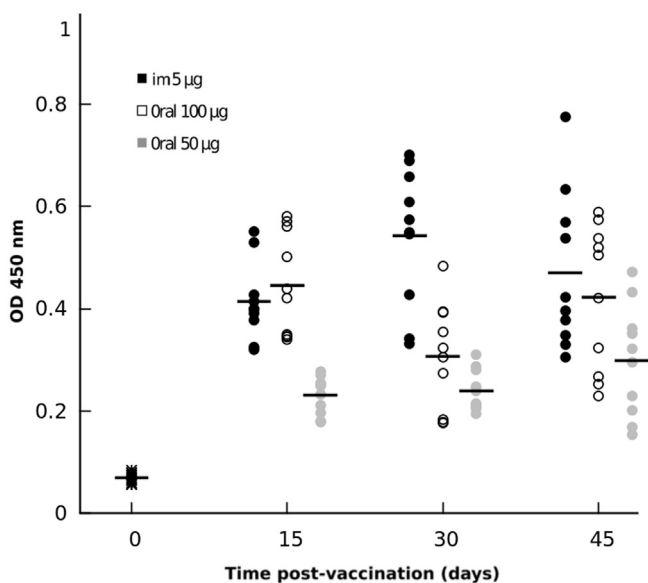
Fig. 7. Alginate-encapsulated pIRF1A-G vaccine induced the production of anti-IHNV antibodies in serum of vaccinated fish. At 15, 30 and 45 days p. v. and before challenge with IHNV, blood samples were collected from the caudal vein of fish orally vaccinated with 50 and 100 μg of pIRF1A-G, and from fish injected with 5 μg of pIRF1A-G. The corresponding serum samples were tested for anti-IHNV antibodies by ELISA. The bars represent the mean antibody levels for 10 fish tested at each time point. i.m.: intramuscularly delivered vaccine; zero: unvaccinated fish.

CD8 expression confirming that different vaccine delivery routes stimulate different cellular immune responses.

Until recently, teleost fish B cells were thought to express only two classes of immunoglobulins, IgM and IgD, in which IgM was thought to be the only Ig responding to pathogens both in systemic and mucosal compartments. However, a third teleost immunoglobulin class IgT, similar to zebrafish IgZ, appears to play an essential role in gut mucosal immune responses [34]. Previously, we analysed local production of Igs in one of the fish intestinal segments where mucosal immunity is induced after oral vaccination, the pyloric caeca [42]. Increased IgM and IgT expression levels in pyloric ceca compared to kidney were detected after the oral administration of alginate microparticles containing pcDNA-VP2 DNA vaccine [17]. In our current study, we measured IgM and IgT production in head kidney and spleen after the oral or intramuscular administration of pIRF1A-G. In teleost fish, the head kidney is considered the primary lymphoid tissue, a key hematopoietic organ, and an important source of Ig-secreting B cells [14]. The spleen, like the head kidney, also contains a large number of B cells and is an organ in which activation and differentiation of B cells occur. Our results showed differential modulation of IgM and IgT 15 days after the oral or intramuscular delivery of pIRF1A-G. While the oral administration of 100 μg of pIRF1A-G vaccine resulted in a significant induction of both IgM and IgT gene expression in kidney and spleen of the vaccinated fish, the intramuscular delivery of the vaccine only induced significant levels of IgT expression in spleen of vaccinated fish. No significant production of IgT was detected in kidney of the i. m. injected fish, confirming IgT up regulation only when the vaccine was orally administered. In agreement with these results, no significant differences in IgM production were detected in serum samples from intramuscular and oral vaccinated fish at the three assessed p. v. time points. It should be pointed out that the efficient induction of a local immune response in the GALT after oral vaccination was shown to induce a more intense immune response than the systemic immunization [14,43]. Therefore, the detection of antibodies in the gut mucus after the administration of oral DNA vaccines should be a matter of further evaluation. In addition, further research is needed to understand the role of the mucosa-associated IgT in the teleosts's gut.

The effect of five doses of the oral pIRF1A-G vaccine on fish CPM was evaluated. Fish injected with 5 μg of pIRF1A-G were used as a positive control group. Dose response was evident for the oral pIRF1A-G treatment groups. Statistically significant differences in mortalities between the fish orally vaccinated with 10, 20, 25 and 50 μg of the oral DNA vaccine and the empty-plasmid vaccinated or unvaccinated fish were observed. The oral administration of 100 μg of pIRF1A-G encapsulated in alginate microparticles to rainbow trout protected against the infection, reaching an RPS value of 56, which is less than the RPS obtained when 5 μg of pIRF1A-G vaccine were i. m. injected (RPS = 70). However, in a previous study, the i. m. administration of 5 μg of pIRF1A-G resulted in protection levels up to 55 RPS [8]. RPS differences between both studies might be explained by differences in mortalities in the control treatment groups. Other authors previously assayed oral DNA vaccines against IHNV encapsulated by the polymer poly-(D,L-lactic-coglycolic acid) (PLGA) and added to feed [44]. The induced protection was much lower than that achieved with the intramuscularly injected vaccine and was also lower than that described here for the alginate-encapsulated pIRF1A-G vaccine. However, both alginate and PLGA carrier systems provide accurate plasmid coating, as persistence of transgene expression was observed in several tissues after vaccination.

In summary, our results demonstrated that alginate microspheres protected the pIRF1A-G vaccine which was expressed in several organs of vaccinated trout. A low dose of the oral vaccine

(10 μg) was able to stimulate the expression of IFN-1, TLR-7 and IgM gene in kidney of orally vaccinated fish. In addition, the oral administration of 10 μg of pIRF1A-G vaccine resulted in a significant induction of Mx-1, Vig-1, Vig-2 and TLR-3 gene expression at 3 d p. v. in spleen of vaccinated trout. In contrast, significant Mx-3 and TLR-8 gene expression was only detected when high doses of pIRF1A-G vaccine were orally administered. High doses of the oral DNA vaccine (50 and 100 μg) induced anti-IHNV antibodies and protected 45 and 56% of the vaccinated trout, respectively. However, there is still need for improvement of this oral IHNV vaccine. An approach to reduce the amount of pIRF1A-G required for oral vaccination and to increase its immunogenicity might be the co-administration of encapsulated mucosal adjuvants such as traditional aluminium salts, polysaccharides (e. g. zymosan, glucans, chitosan), and TLR agonists [5,45]. Moreover, the oral administration of plasmids encoding cytokine adjuvants (IL-8, IL-2, IFN-γ, IL-12, GM-CSF and IL-15) may be another approach to increase the immunogenicity of the oral pIRF1A-G vaccine [45,46]. As CD8+ cells dominate the CD4+ subset in the intestine, oral DNA vaccines should be developed to specifically target these effectors immune cells at the mucosal surfaces as a way to increase their efficacy. Induction of long-term memory by specific stimulation of mucosa-associated IgT should also be considered. Perhaps such improvements could also increase the efficacy of the oral pIRF1A-G vaccine in terms of survival (>56 RPS). In other hand, it remains to be determined whether the addition of the oral pIRF1A-G vaccine to food pellets could serve as an efficient method of inducing protective immune responses against IHNV infection. Using this approach, the plasmid could be easily delivered in multiple successive events, avoiding fish stress and likely increasing vaccine effectiveness. Previously, we demonstrated that oral vaccine delivery through feeding is a promising delivery method, at least for IPNV vaccines [19]. In conclusion, our results demonstrated a significant increase in fish immune responses and resistance to an IHNV infection after the oral administration of increasing concentrations of a DNA vaccine against IHNV encapsulated into alginate microspheres.

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