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Food pellets as an effective delivery method for a DNA vaccine against infectious pancreatic necrosis virus in rainbow trout (*Oncorhynchus mykiss*, Walbaum)

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ABSTRACT

A DNA vaccine based on the VP2 gene of infectious pancreatic necrosis virus (IPNV) was incorporated into feed to evaluate the effectiveness of this oral delivery method in rainbow trout. Lyophilized alginate–plasmid complexes were added to feed dissolved in water and the mixture was then lyophilized again. We compared rainbow trout that were fed for 3 consecutive days with vaccine pellets with fish that received the empty plasmid or a commercial pellet. VP2 gene expression could be detected in tissues of different organs in the rainbow trout that received the pcDNA-VP2 coated feed (kidney, spleen, gut and gill) throughout the 15 day time-course of the experiments. This pcDNA-VP2 vaccine clearly induced an innate and specific immune-response, significantly up-regulating IFN-1, IFN-γ, Mx-1, IL8, IL12, IgM and IgT expression. Strong protection, with relative survival rates of 78%–85.9% were recorded in the vaccinated trout, which produced detectable levels of anti-IPNV neutralizing antibodies during 90 days at least. Indeed, IPNV replication was significantly down-regulated in the vaccinated fish 45 days pi.

1. Introduction

The control of infectious diseases is essential to maintain the levels of productivity in the aquaculture industry. The economic impact of infectious diseases, particularly those of viral aetiology, is a constant worldwide threat in the salmonid fish industry, stimulating research to find efficient methods to minimize such losses. Vaccination is the most effective approach to combat disease in aquaculture, a strategy that is ideal to prevent and avoid the dispersion of infective viruses in fish, particularly in farms where fish are raised under intensive culture conditions. Although different types of viral vaccines have been described for fish, including inactivated, attenuated, synthetic peptides or subunit vaccines [1–3], protection is not always complete. Hence, studies are necessary to produce improved vaccines capable of inducing longer lasting immunity and less stressful methods of administration [4]. Genetic vaccines were first developed for mammals in the 1990s and several designs to protect against rhabdoviruses have been tested in salmonid fish species [5–12]. More recently, other DNA vaccines have been described to combat the infectious pancreatic necrosis virus (IPNV), another viral pathogen of salmonid fish [13–15].

Infectious pancreatic necrosis virus (IPNV) is the type species of the Aquabirnavirus genus, from the *Birnaviridae* family [16]. Virions are non-enveloped and they contain two segments (A, B) of double-stranded RNA. Segment A is the larger of the two (about 3.1 kbp) and it encodes VP2 and VP3, the two major structural proteins of the virus [17,18]. The VP2 protein is the type-specific antigen that can induce the production of neutralizing antibodies that are capable of protecting susceptible fish from viral infection [19–21]. IPNV is one of the main causes of mortality worldwide for juvenile salmonid fish, being especially destructive in salmonid eggs and fingerlings [22]. Fish surviving IPN epizootics develop a persistent viral infection or carrier state, capable of continually transmitting the virus to other susceptible populations of fish, including their own offspring [23–26] (for reviews see Refs. [22,27,28]).

Genetic vaccination for IPNV has only recently been undertaken experimentally, and the initial steps in its development have focused on traditional injection methods [13]. Intramuscular injection of DNA vaccines has been successfully used against viruses such as infectious haematopoietic necrosis virus (IHNV) or viral haemorrhagic septicaemia virus (VHSV). Intraperitoneal injection has also been routinely used for other vaccines, such as recombinant vaccines and multivalent products, and automated systems for

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injection have been developed [3]. However, oral vaccines would be easier to administer and they represent an important alternative to immunize fish against viruses. Nevertheless, there are still few reports of successful vaccine delivery methods other than injection, with delivery in feed having been mostly licensed to prevent bacterial but not viral diseases [4].

We previously described a DNA vaccine derived from the VP2 gene of IPNV inserted into an expression plasmid and encapsulated into alginate microspheres. The oral delivery of the plasmid (diluted in PBS) was performed manually in order to ensure the uniform vaccination of the fish under study. Strong protection was achieved in this way, with around 83% relative survival when challenged 15 and 30 days after vaccine delivery. Indeed, strong expression of IFN and the IFN-induced antiviral Mx protein was recorded 7 and 15 days post-vaccination (pv) [14,29]. However, novel approaches to improve the efficacy of DNA vaccine oral delivery would not only provide interesting data regarding the future mass delivery of these vaccines but also, keys to understand the cellular and mucosal immunity reactions. Oral delivery of DNA vaccines is a process that has been poorly explored, especially against IPNV. Thus, having generated a vaccine that successfully induces appropriate immune protective responses, the next step should be to check if this vaccine can be delivered in feed without losing its beneficial effects due to the severe conditions experienced during gastrointestinal transit.

The goal of the present work was to determine the effectiveness against IPNV of the pcDNA-VP2-encapsulated in alginate and incorporated into fish feed, and the immune responses it induces in rainbow trout. Given that this method appears to produce similar results to those described previously, its potential should be further assessed for industrial application.

2. Materials and methods

2.1. Ethics statement

The experiments described comply with the Guidelines of the European Union Council http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm (Directive 2010/63/EU) for the protection of animals used for scientific purposes and were previously approved by the CSIC Ethics committee.

2.2. Cells and virus

The BF-2 cell line from bluegill fry (*Lepomis macrochirus*, ATCC-CCL 91) was used to isolate and propagate the viruses. The IPNV Sp strain from the ATCC was used in this study (ATCC VR 1318), and all the cells and viruses were cultured as described previously [30]. Briefly, cells were grown at 25 °C in Leibovitz's medium (L15, Gibco, Spain) supplemented with 100 IU mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine and 10% foetal bovine serum (FBS: Gibco, Spain), or with 2% FBS in the maintenance medium (MM). The virus was cultured in L-15 medium and propagated at 20 °C. The virus was titrated in 96-well culture plates (Falcon, Becton–Dickinson) infected with 10-fold serial dilutions and the plates were observed daily for the development of a cytopathic effect (CPE). The infective titres were determined as the 50% infective dose in tissue culture ($TCID_{50}$ mL^{-1}): based on Reed & Muench [31].

2.3. Fish

Rainbow trout (3.5–4 cm and 1.5 g mean size and weight) were purchased from a local spring water farm with no history of viral disease. The fish were kept at the "Centro de Investigaciones Biológicas" (CSIC, Madrid, Spain) under a 12/12 h light/dark regime at 15 °C in 350 L closed re-circulating water tanks (Living Stream, Frigid Units Inc, Ohio). The fish were fed daily with a diet of commercial pellets and they were maintained as described elsewhere [32]. To assess their health, pools of five fish were examined for viruses by standard protocols [33,34], none of the fish lots examined giving positive results. The trout were anaesthetized with buffered tricaine methanesulphonate (MS-222, Sigma) prior to handling and the experiments described comply with the European Union Guidelines (86/609/EU) for the use of laboratory animals.

2.4. Oral vaccination

2.4.1. Vaccine and preparation of fish feed

The pcDNA-VP2 plasmid in *Escherichia coli* (TOP10) was prepared as described previously [14]. Cultures were grown in 10 L of LB broth and the cells were then recovered by centrifugation and frozen at −20 °C. The plasmid was purified from the cells using the QIAGEN plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. The pcDNA-VP2 and pcDNA plasmids were coated with sodium alginate and these microspheres were prepared as described previously [29]. The feed (T-2.0 Nutra from Trow España S.A, Burgos, Spain) was that recommended for fish that are approximately 3.5 cm long and that weigh 1.5 g, and it was the same as that used at the farm that provided the rainbow trout. This feed contains 54% protein from fish-meal, 18% oil, 1% cellulose, 11.5% ash, 3% calcium, 1.2% sodium and 1.7% phosphorus, as well as several other oligo-elements and anti-oxidants. The particle size ranged from 1.0 to 1.7 mm.

In our previous studies with vaccine–alginate complexes, the fish received daily drops containing 10 μg vaccine. In the present work, the size and weight of the vaccinated trout were similar (3.5 cm, 1.5 g) and since the fish are thought to ingest around 5% of their body weight daily, each fish should receive 0.075 g of feed/day. Thus, the experimental vaccine was prepared in lots of feed for 120 fry trout by placing commercial dry pellets (27 g) into 50 ml Falcon tubes and along with 3.6 mg of pcDNA-VP2-alginate microspheres previously diluted in 15 ml of distilled water. The pellets and microspheres were then mixed gently for a few minutes at room temperature, and the middle-moist feed obtained was lyophilized for 24–48 h and conserved at 4 °C until it was used. In this way, a vaccine concentration of 10 μg per fish and day was achieved.

2.4.2. Fish vaccination

Groups of 25 trout were placed in separate 40 L aquaria maintained at a constant temperature of 15 °C for treatment. The first group of fish was vaccinated by providing with vaccine impregnated food pellets at 5% of body weight for three consecutive days (10 μg pcDNA-VP2/fish/day). The second group of rainbow trout was fed with pellets mixed with the empty pcDNA plasmid, serving as the plasmid control, and the third group of fish received the commercial pellets and was considered as the untreated mock vaccinated fish control. An additional group of fish were vaccinated individually with a pcDNA-VP2 alginate microspheres solution in water, and used as a positive control in the light of the results obtained with this method elsewhere [29].

2.4.3. Tissue distribution and time-course of pcDNA-VP2 expression

At 1, 3, 5 and 15 days pv (after the last feed), 3 trout from each group were sacrificed with an overdose of MS-222, and the kidney, spleen, liver, gut and gill tissue was removed aseptically. Total RNA was isolated using the TRIzol reagent (Invitrogen, Spain), according to the manufacturer's instructions, and treated with DNase I to remove any trace genomic DNA that might interfere with the PCR reactions. Equal amounts of RNA were primed with oligo(dT) and

cDNAs were synthesized to analyse the VP2 gene expression by real-time PCR as described previously [14,34,35].

2.4.4. Gene expression

At days 1, 2, 3, 5 and 7 pv, trout were sacrificed ($n = 3$) and the head kidneys were processed for RNA extraction. We analysed the expression of five genes by RT-qPCR, three belonging to interferon-related pathways (IFN-1 [36], IFNg [37] and Mx-1 [38–40] and two interleukins IL8 [41] and IL12 [42]). On days 15 and 30 pv, the kidney of trout ($n = 3$) from each group was also processed to evaluate the expression of genes related to adaptive immune responses, such as IgM [43] and IgT [44]. All the primers used had been reported and optimized in previous studies [34,35]. The transcription of CD4 and CD8 Th cell markers was also assessed using the primers CCTGCTCATCCACAGCCTAT (F) and CTTCTCCTGGCTGTCTGACC (R) for CD4 and AGTCGTGCAAAGTGGGAAAG (F) and GGTTGCAATGGCATACAGTC (R) for CD8. The corresponding accession numbers are AY973030.1 and NM_001124263, respectively. All qPCR reactions were performed in triplicate and for each mRNA, and gene expression was normalized to that of the endogenous control (elongation factor 1-α; EF1-α) and expressed as $2^{-\Delta Ct}$, where ΔCt was determined by subtracting the average EF1-α Ct value from the average target Ct. The change in expression relative to the empty plasmid pcDNA was also determined for some of the samples by applying the formula $2^{-\Delta\Delta Ct}$ [45] where ΔΔCt = ΔCt of samples of target gene −ΔCt of the calibrator (pcDNA control).

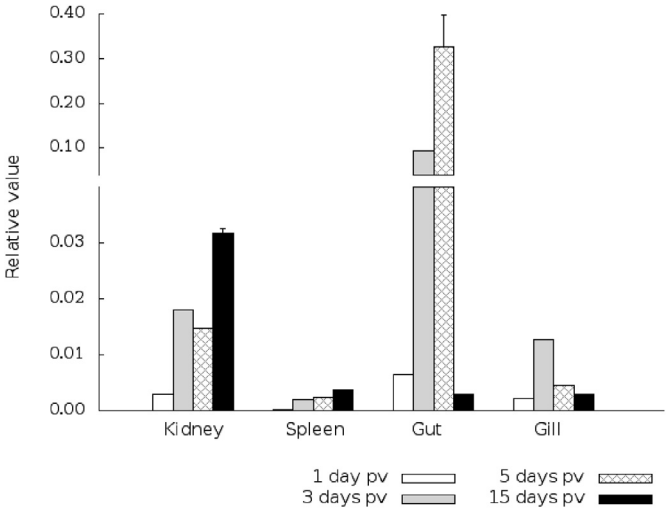


Fig. 1. Relative quantification of VP2 gene expression assessed by real-time PCR. Total mRNA was obtained from different tissues (head kidney, spleen, gut, and gill) at 1, 3, 5 and 15 days post-vaccination with pcDNA-VP2. The vaccine was incorporated into commercial pellets and the fish were fed the DNA vaccine diet on three consecutive days. The first day without feeding vaccine pellets was considered day 1 post-vaccination. The data are shown as the mean gene expression relative to the expression of the endogenous control EF-1α ($2^{-\Delta Ct}$ method). The error bars represent the St. dev. of the mean ($n = 3$).

2.5. Titration of neutralizing antibodies

A neutralization test (NT) was used to evaluate the specific immune response against IPNV in the vaccinated trout. Fish blood was collected by caudal puncture at 15, 30, 60, and 90 days pv, and the IPNV neutralizing antibody titre was analysed in the serum of individual fish. This assay involved incubating a two-fold dilution of the serum with a known amount of reference IPNV, and the mixtures were then assayed in triplicate on BF-2 cells. Similarly, IHNV was also tested to assess the specificity of the reaction. After incubating for 3 days, the cell cultures were fixed and stained with a crystal violet solution (2% in ethanol). The titre of a serum was determined as the reciprocal of the serum dilution that reduced viral infectivity by approximately 50% when compared to the virus control ($TCID_{50}$ mL^{-1}). Titres >40 were considered positive.

2.6. IPNV challenge

To examine the efficacy of the DNA vaccine, the vaccinated and control groups of fish were challenged with IPNV and monitored for cumulative mortality. Rainbow trout were divided into three groups (25 fish each) and were vaccinated by feeding with pellets containing the encapsulated pcDNA-VP2 plasmid on 3 consecutive days (10 μg DNA/fish/day: group 1). A second group 2 received similar amounts of pellets impregnated with the encapsulated empty pcDNA plasmid and the third group of fish was fed with commercial pellets. An additional group of fish were vaccinated individually with a pcDNA.VP2 alginate microspheres solution in water, and used as a positive control in the light of the results obtained with this method elsewhere. Water quality was maintained at optimum levels and all tanks were kept under equivalent conditions. On day 15 or 30 pv, each group of fish were challenged with IPNV by immersion (1×10^6 $TCID_{50}$ mL^{-1}), as described previously [29]. Fish mortality in each group was subsequently recorded and any dead fish were removed daily over the next 30 days.

Vaccine efficacy was determined by comparing the average cumulative percentage of mortality (cpm) and the relative percentage of survival (RPS: [1 − (% mortality in vaccinated fish/% mortality in control fish) × 100]). Two replicas of the trial were carried out.

2.7. Expression of the IPNV-VP4 gene in fish after vaccination and IPNV challenge. Isolation of virus from survivors

The expression of the IPNV-VP4 gene was used as a measure of the replication of IPNV in the control fish, and in the vaccinated and infected fish. Three trout from each group were sacrificed with an overdose of MS-222 on day 45 post-infection (pi) and their kidney tissue was removed aseptically. Total RNA was isolated and cDNAs were synthesized to analyse IPNV-VP4 gene expression by real-time PCR (see above). The VP4 gene serves as a marker of IPNV, making it possible to distinguish the VP2 vaccine expression from the IPN viral expression. The relative level of infection in the vaccinated and infected fish was then estimated by RT-qPCR analysis.

The virus was isolated by processing head kidney samples from the same fish assayed by RT-qPCR, and BF-2 cells were inoculated with aliquots of the homogenates as described previously [32].

2.8. Statistical analysis

Prior to performing statistical analyses, the normal distribution of the data was checked and confirmed using the Shapiro–Wilk test. The data are presented as the mean ± standard deviation (St. dev.) of the results from three trout. An analysis of variance (factorial ANOVAs) was run to determine whether the differentially expressed gene differed between the replicates for each individual gene, followed by Tukey's multiple comparison test to assess the differences between the vaccinated and control group. The Student *t*-test was also used to compare some paired samples. All statistics were analysed with the IBM SPSS Statistics package, an integrated family of products that addresses the entire analytical process, from planning and data collection to analysis, reporting and deployment

(IBM® SPSS® Statistics 15; http://www.spss.com). $P \leq 0.05$ was considered significant.

3. Results

3.1. Tissue distribution of the VP2 gene

To assess whether the DNA vaccine delivery approach tested may be suitable for immunization against IPNV, the tissue distribution of the VP2 gene was first studied. The presence of mRNA-VP2 was quantified in several organs over time and in the kidney of the vaccinated fish, and VP2 gene expression increased strongly from 3 days to at least 15 days pv. When compared to the fish tested on day 1, relative increases in expression of around 4.7 fold and 12.5 fold were detected at 3 and 15 days pv, respectively (Fig. 1). The spleen was the organ where the weakest relative expression of VP2 was recorded at any of the time points selected. The strongest expression of VP2 was recorded in the gut with an increase at 3 days and with a peak of expression on the 5th day, when the relative expression was around 52-fold that on day 1. However VP2 expression in the gut decreased dramatically to basal levels on the 15th day. This VP2 transcript was also detected in the gill, although again at lower levels.

3.2. Transcriptional changes of immune-related genes in the kidney of rainbow trout 7 days post-vaccination

We previously used a specific microarray to analyse the genes expressed by rainbow trout 7 days pv with the pcDNA-VP2 [34]. Some of the genes found to be differentially expressed in the vaccinated fish, and/or induced during IPNV infection of trout, were also studied here. These expression profiles were evaluated to demonstrate that oral delivery of the plasmid and VP2 expression induced innate and adaptive responses.

Administration of pcDNA-VP2 in the feed induced a strong increase in the expression of the genes selected to reflect an innate-immune response to the virus. The increase in expression of the marker genes induced in vaccinated fish is shown relative to the fish that received pellets containing the empty plasmid (pcDNA: Fig. 2). The increase in expression induced by the pcDNA-VP2 vaccine was strongest for IFN-1 and IL12, with values ranging from 57 to 82-fold, while the expression of the Mx-1 and IL-8 was also up-regulated on day 3 pv (25- and 47-fold, respectively). The expression of all the genes tested was significantly up-regulated for several days, with the highest increases observed on the second to the third day pv. The significant up-regulation of IL-12 gene expression persisted throughout the experiment and it was approximately 13-fold at 7 days pv. By contrast, the mRNA expression of all the other genes decreased to near basal levels at 7 days pv.

3.3. Transcriptional changes in several adaptive immune-related genes in the kidney of rainbow trout at 15 and 30 days post-vaccination

Of the many genes involved in the specific immune-response we choose to study: i) the IgM gene that is related to cellular immune responses; ii) IgT, recently associated with mucosal immunology; and iii) the expression of the CD4 and CD8 genes, T-cell markers, considered to play an important role in eliminating virus-infected cells. The changes in gene expression in vaccinated fish relative to the pcDNA group of fish were assessed (Fig. 3) and the strong IgM and IgT expression detected in the kidneys 15 days pv increased significantly at 30 days pv (around 17- and 11-fold, respectively). By contrast, appreciable levels of CD4 and CD8 genes were recorded but no significant changes were observed at the times assayed.

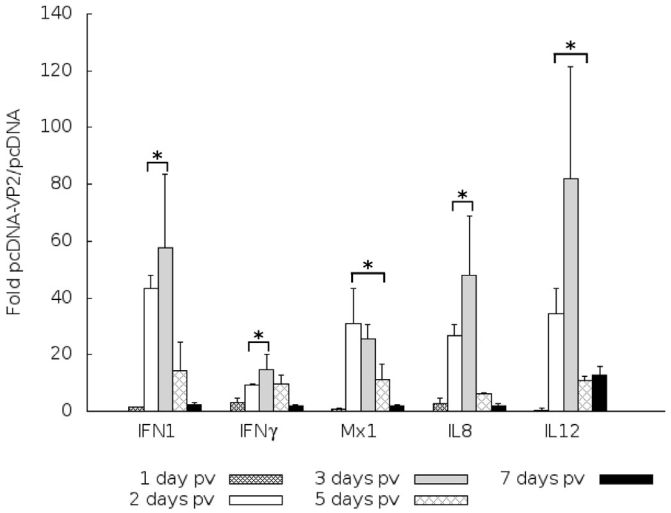


Fig. 2. The change in expression of immune innate-related genes assayed by RT-qPCR in the rainbow trout kidney at 1, 2, 3, 5 and 7 days post-vaccination with pcDNA-VP2 plasmid incorporated into the diet. The vaccine was administered on three consecutive days and the first day without feeding vaccine pellets was considered day 1 post-vaccination. The head kidney tissue from 3 fish was collected and the RNA extracted for RT-qPCR analysis, which was performed in triplicate. The endogenous EF-1α gene was used to normalize the results and the data are presented as the mean fold increase relative to fish fed with the pcDNA empty plasmid pellets (±standard error: $2^{-\Delta\Delta Ct}$ method). Asterisks indicate significant differences ($P \leq 0.05$) relative to the data from day 1.

3.4. Neutralizing antibodies

The anti-IPNV neutralizing antibodies were examined in serum samples from fish at 15, 30, 60 and 90 days pv (Table 1). Anti-IPNV

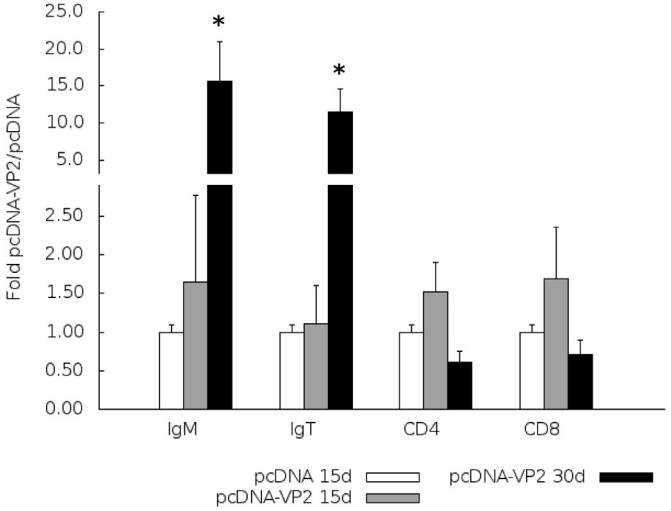


Fig. 3. The change in expression of IgM, IgT, CD4 and CD8, adaptive-related immune genes, assayed by RT-qPCR in rainbow trout kidney 15 and 30 days after dietary pcDNA-VP2 vaccination. The vaccine was administered on 3 consecutive days and the first day without feeding vaccine pellets was considered day 1 post-vaccination. A second group of fish received the food pellets impregnated with the empty pcDNA plasmid. Total mRNA was obtained from the head kidney and the fold increase in transcripts compared to the empty plasmid controls was assessed. The error bars represent the St. dev. of the mean ($n = 3$) and the asterisks indicate statistically significant differences relative to the control at 15 days pv ($P \leq 0.05$).

neutralizing antibodies were detected in 18 of 20 fish in the group fed with pcDNA-VP2 and tested at 15 and 30 days pv, whereas all the fish tested at 60 and 80 days had anti-IPNV.VP2 antibodies. Neutralizing titres were higher in these late groups, from 160 to 640. No antibodies against IPNV were detected in the sera from the control fish (saline solution administered) and fish that received the empty plasmid pcDNA. In these studies a titre ≥40 was defined as positive and low titres of anti-IPNV neutralizing antibody were only observed in 3 of the 40 total vaccinated fish.

The specificity of the induced antibody was demonstrated by the failure to neutralize IHNV. A progressive increase in the anti-IPNV neutralizing antibodies was recorded, demonstrating the antigenic potential of the VP2 gene.

3.5. Cumulative mortality and relative percent survival (RPS) after IPNV challenge to vaccinated fish

When rainbow trout were analysed 15 or 30 days pv and challenged with IPNV (Fig. 4), a cumulative mortality of 83% was observed in the challenged control fish (A: IPNV control), as well as in the fish fed pellets containing the empty pcDNA plasmid (B). When the kinetics of mortality were analysed in this later group, it was slightly retarded with respect to the virus control group until the 20th day post-challenge when the mortality rates were similar. By contrast, the fish vaccinated via the feed were strongly protected, with a relative percent survival (RPS) of around 86%. Similarly and as expected, there was strong protection of fish that received the vaccine individually as a microspheres emulsion delivered by pipette (RPS = 73–76%), in which the onset of mortality was also delayed when they were challenged at 30 day pv. However, in this particular group the cumulative mortality profile was the highest of all the groups vaccinated in these experiments. The fish vaccinated individually or through the feed, and that were challenged on the 15th day pv, exhibited parallel mortality curves from 15 to 25 days post challenge. Likewise, similar proportions of these fish died, and they also resisted the challenge better than the corresponding groups challenged at 30 days pv. Nevertheless, at the end of the experiment the strongest protection was induced in the fish administered the vaccine with the feed and that were challenged at 15 or 30 days pv (85.9% and 78.2% RPS, respectively), reflecting the efficiency of vaccination by this route of administration.

3.6. Expression of the VP4 gene from IPNV at 45 days post-challenge and the isolation of the virus from survivors

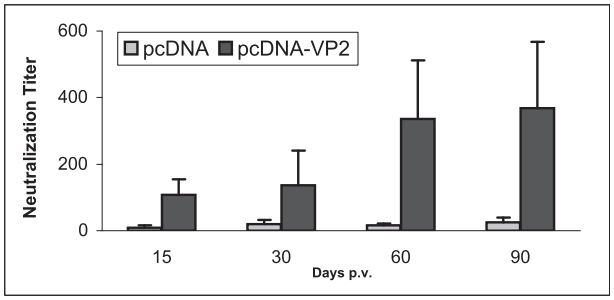
Viral gene transcription that involved the VP4 gene was analysed by real-time PCR using RNA from the head kidney as the template to evaluate viral load. The expression of the gene encoding the IPNV-VP4 protein was determined by RT-qPCR in the fish that survived 45 days post-challenge, those infected control fish group ($n = 3$), as well as in the vaccinated and challenged fish ($n = 3$). The VP4 gene was examined so as to distinguish between vaccinated and infected trout (Fig. 5), and its expression in the virus control group was 7-fold greater than in the vaccinated and infected fish in which there was a significant reduction in expression. These results suggest that IPNV replication was significantly down-regulated or that very low levels of the virus were found in the vaccinated fish 45 days pi.

4. Discussion

The present study set out to determine if addition of an IPNV-DNA vaccine to food pellets could serve as an efficient method of vaccine delivery, inducing an immune response and protecting rainbow trout against IPNV infection. Accordingly, we demonstrated that this vaccine was taken up by the fish and that the plasmid passed across the gut barrier, allowing the transcript to be efficiently expressed. The time-course experiments over 15 days confirmed the expression of the antigenic gene in all the organs examined. Among these, vaccine expression was strongest in the kidney for at least the first 15 days pv, while the expression in the gut peaked at 5 days pv but decreased to basal levels at 15 days pv. This profile could be explained by the abundant presence of the

Table 1
Neutralizing antibody titres in sera samples from fish immunized with the vaccine or the pcDNA plasmid in the feed.

Samples	Days post-vaccination			
	15	30	60	90
pcDNA-VP2[a]	80	320	320	160
	160	80	160	640
	80	40	320	160
	160	80	640	640
	80	80	640	320
	160	320	320	160
	160	40	160	640
	80	80	320	320
	40	160	160	320
	80	160	320	320
	<20	<20	<20	<20
pcDNA-VP2[b]	<20	<20	<20	<20
	<20	<20	<20	<20
	20	40	20	40
	0	20	20	10
pcDNA[a]	10	5	20	20
	10	20	10	20
	5	10	10	40
Saline solution[a,b]	0	0	0	0



pcDNA-VP2: Sera from fish fed with plasmid that encodes the IPNV VP2 gene; pcDNA: Sera from fish fed with empty plasmid.
Different fish were examined from 15 to 90 days post-vaccination. The antibody titre is the reciprocal of the highest dilution of the antiserum protecting against the standardized virus.
The histograms show mean values of neutralization titres against IPNV. Bars represent standard deviation.
[a] Sera tested against IPNV.
[b] Sera tested against IHNV.

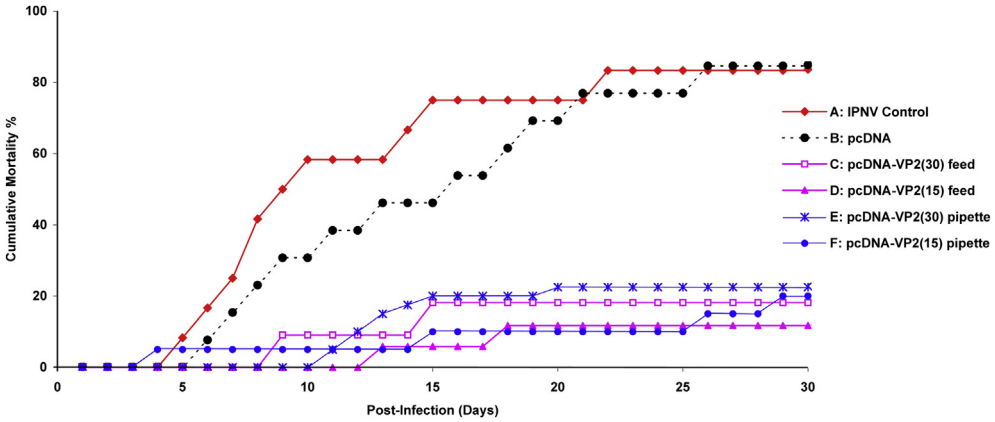


Fig. 4. Cumulative mortality of rainbow trout (1.5 g mean weight) challenged by immersion with 5×10^5 $TCID_{50}$ mL^{-1} of IPNV. The fish ($n = 20$ in duplicate tanks) were (A) mock-vaccinated with commercial food pellets and infected with IPNV, (B) mock-vaccinated with the empty pcDNA plasmid and infected with IPNV; (C, D) fed pellets incorporating the pcDNA-VP2 vaccine on 3 consecutive days and infected with IPNV; (E, F) pcDNA-VP2 vaccine individually administered by pipette tip. The fish were challenged on post-vaccination day 30 (C and E) and 15 (D and F), and monitored for up to 30 days.

plasmid soon after administration and the progressive passage across the intestinal barrier into the bloodstream, as suggested for other vaccines [46].

The expression of some representative genes was assayed to determine whether the vaccine was eliciting an immune response.

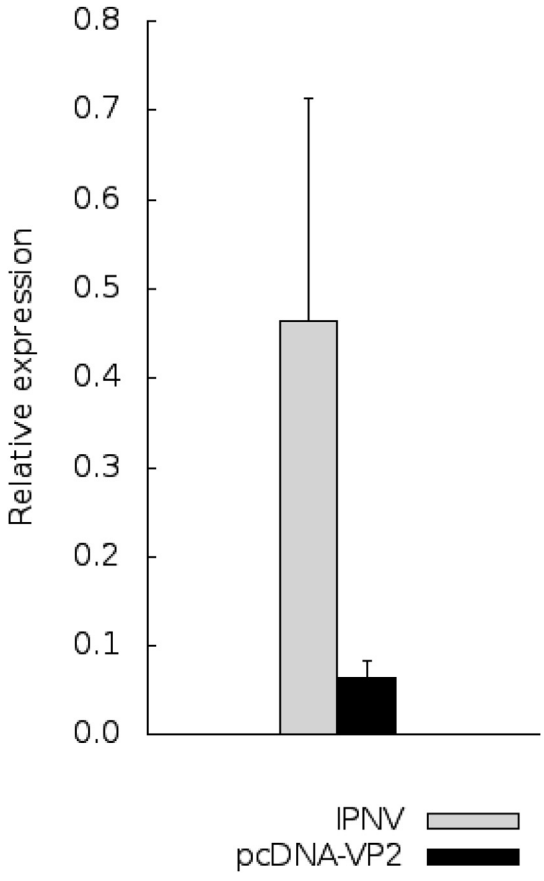


Fig. 5. Relative expression of the IPNV-VP4 gene in the IPNV infected fish, and in the vaccinated and challenged fish group 45 days post-challenge. The fish were vaccinated with pcDNA-VP2 through the diet on 3 consecutive days and they were then challenged with the virus (5×10^5 $TCID_{50}$ mL^{-1}) after 30 days. A second group was mock-vaccinated and infected with IPNV (virus control group). Transcription of the VP4 gene was recorded in the kidneys of both vaccinated and virus control fish at 45 days post-challenge, and the data are represented relative to EF1-α expression ($2^{-\Delta Ct}$ method), (mean + S.E., $n = 3$). Values with asterisks are significantly different ($P \leq 0.05$).

The transcriptional profile of genes involved in the immune response in the trout head kidney was determined previously by microarray hybridization after oral administration of the vaccine (plasmid–alginate complexes in water, administered individually through a pipette tip) [34]. From the large number of the genes up-regulated, fourteen of those related to immune responses were selected for quantitative RT-qPCR validation. In the present work, several IFN and interleukin-related genes, as well as the IgM and IgT genes, from that study were selected to analyse the time-course of their expression in the head kidney. These genes were considered to be suitable markers to assess the immunization procedure related to both the innate and specific responses. Other immune markers, such as those of the CD4 and CD8 T-cell lineage, have only recently been characterized in salmonids and their expression was determined here.

Among the non-specific immune events, it is well established that fish can secrete type I interferon in response to viral infection and it is one of the gene markers used to studying the early protection induced by a vaccine. Our results clearly show that the pcDNA-VP2 vaccine up-regulates IFN-1 expression, with early increases of around 57-fold with respect to the empty plasmid. A comparison with previous results with the alginate coated vaccine [29] suggests that the expression of the innate-immune-related genes was clearly higher in the fish fed with pcDNA-VP2 vaccine pellets. As expected, IFN-1 was expressed most strongly 2–3 days pv, although significant up-regulation of IFN-γ was also evident after 5 days (also named IFN-II), as also observed at different times for Mx-1, IL8 and IL12 in the vaccinated fish with respect to the fish fed with the empty plasmid. In turn, fish IFN II can induce the expression of many interferon induced genes (ISGs) that also respond to type I IFNs, suggesting cross-activation of the innate antiviral responses elicited by type I and II IFNs. Our results showed that the VP2 vaccine also modulates pro-inflammatory cytokines like IL-12, which was significantly up-regulated at 2, and even 7 days pv. Moreover, interleukin 8 (IL-8) was also rapidly up-regulated, a gene included in the CXCa group of chemokines, a family of cytokines that regulate immune cell migration under both inflammatory and normal physiological conditions. These genes not only promote leukocyte mobilization but also, they regulate the immune responses and differentiation of the recruited cells. Accordingly, they have been catalogued as key regulators of the immune response, acting as a bridge between the innate and adaptive responses [47]. Strong IL8 expression has been also reported following IHNV and VHSV infection, suggesting a role for this cytokine in viral defence [48,49].

We also evaluated the expression of genes representative of the adaptive immune response, immunoglobulins (Ig) M and T, and the Th cell markers CD4 and CD8. In mammals, Igs can be divided into five categories and for a long time, it was believed that only one of them, IgM, existed in fish. However several other functional Igs have recently been discovered in teleost fish and Ig genes were identified in rainbow trout, named IgT, similar to zebra fish IgZ [50]. It was also recently reported that rainbow trout contained a new B linage uniquely expressing surface IgT [44]. It is interesting that genes related to adaptive immunity were already being expressed by the 7th day after the last feed with the vaccine (the 10th day after the first feed), which could be relevant for the effectiveness of the vaccine.

We recently studied immune responses in the rainbow trout gut and pyloric caeca region, the area in which a major recruitment of B cells was demonstrated [51]. Moreover, we found significant increases in the number of both IgM^+ and IgT^+ intraepithelial lymphocytes after manual oral vaccination with the pcDNA-VP2 vaccine. In the present work, the head kidney was the target organ assayed, considered to be the primary lymphoid tissue in teleost fish and thus, an important source of B cells. The IgM and IgT genes were evaluated here at 15 and 30 days pv when the adaptive immune responses may be more apparent. Interestingly, the expression of both these genes peaked 30 days pv, suggesting the presence of Ig secreting cells, and corroborating the vaccine activity and the gene response to oral stimulation.

The level of expression was also evaluated for other genes, such as CD4 and CD8, although the transcription of these T cell genes was not significantly enhanced. This might be explained by differences between fishes and their ingestion ability, as well as the times selected for the assay. IFN-γ is mainly produced by $CD4^+$ cells in mammals and this gene was significantly up-regulated at 7 days pv, suggesting an activation induced by the vaccine. Further sampling of other organs and assessing the time-course of expression will be necessary to study the mechanisms and pathways that an oral vaccine could initiate in more depth. Here, we only aimed to assay the viability of the feed-delivery method to induce a variety of immune responses. Nevertheless, we demonstrated that VP2 is expressed in organs and that it orchestrates immune responses.

A detailed comparison with the results we obtained previously with an oral vaccine cannot be carried out, since the vaccine–alginate complexes were administered individually on only one dose [34,35] while here the fish were fed with the vaccine over 3 consecutive days. Nevertheless, similar profiles of expression were observed over the first 3–7 days using both methods of oral administration, although the response was stronger when the vaccine was administered in the feed. Peaks of expression were recorded around the 7th day post-vaccination with a pipette, and on the 3rd day (more-or-less) in the present study, which is 6 days after the fish received their first feed with the vaccine. With regards IgM and IgT expression, data were only recorded after the first 8 days post-vaccination in the previous study, when there was only weak induction of these genes ($\leq$3-fold). Here these antibody responses were examined at 15 and 30 days post-vaccination, considered as markers of adaptive immune response, when values around 15-fold were recorded. With regards protection, both methods to deliver the pcDNA.VP2 vaccine diminished the mortality from the very first days post-challenge, which may be related to the innate-immune response induced. Moreover, strong specific protection was evident 30 days post-challenge, as demonstrated by the levels of the neutralizing antibodies recorded and by the cumulative mortality. Taken together, these data show that the pcDNA.VP2 vaccine administered with the feed is stable in the gastric system of the fish, allowing efficient absorption of the antigen, as well as the subsequent induction of innate and adaptive responses.

To fully assess the effectiveness of the vaccine and delivery method, vaccinated and control fish were challenged with virulent IPNV at 15 and 30 day pv, and cumulative mortality was examined over 30 days. The vaccinated trout displayed strong protection, similar levels to those previously described for fish that were individually vaccinated with alginate-encapsulated plasmid [29]. The cumulative mortality was similar in the replicate experiments performed, despite the possible variability in feed uptake over the 3 days of vaccination. Interestingly, very low levels of mortality were recorded during the first 10–12 days in all the vaccinated fish, suggesting an antiviral effect induced by the activation of IFN and other genes involved in immune responses. Mild protection was also initially evident in the pcDNA vaccinated fish, although their final mortality was the same as in the virus control group. In the vaccinated fish the early protective effect seems to be followed by a later phase of specific immunity, as the vaccine induced the production of neutralizing antibodies against IPNV. Since the survival rates recorded are higher when fish were challenged on the 15th day pv, an overlap of both the innate and adaptive protective effects might occur at this time. Despite the role of non-specific defence mechanisms, it is noteworthy how effective the oral DNA vaccine and the VP2 antigen are in progressively inducing the appearance of neutralizing antibodies, and hence, specific protection.

Viral load was quantified by RT-qPCR at 45 days post-challenge in vaccinated virus challenged fish and in virus control trout. Fish from both groups were survivors that displayed no clinical symptoms of disease, yet the expression of the IPNV-VP4 gene detected in the infected non-vaccinated fish was significantly higher than that recorded in those that received the vaccine, suggesting that vaccination reduced viral load. However, PCR detection of a viral gene does not necessarily imply the presence of infective virions, as demonstrated by failure to recover IPNV after inoculation of cell cultures with homogenates from the head kidney from fish (data not shown). Experiments over longer periods than those carried out here would be necessary to determine whether PCR could detect the virus after several months. Meanwhile our results indicate that in the present conditions, the oral vaccine clearly diminished the viral load in the fish. Thus, these results demonstrated that administration through feeding is a promising delivery method, at least for IPNV vaccines.

Few studies on DNA oral vaccines against this or other fish viruses have been performed to date. Some preliminary experiments with an oral vaccine against IHNV did not prove to be as efficient (unpublished data) and a recent study on oral administration of a DNA anti-IHNV vaccine described inefficient protection, although the viability of oral delivery of DNA vaccines was demonstrated [52].

Protection is likely to increase as the doses, encapsulating materials and methods to add the vaccine to the feed improves. The effective uptake of the DNA vaccine and its transcription by enterocytes along the five segments of the digestive tract was suggested in our recent study in which the vaccine was delivered manually as microspheres suspended in solution [51]. The pyloric caeca was the area of the digestive tract in which a major recruitment of B cells was detected and the most responsive to the oral IPNV vaccination of the five segments examined. In this work, the increase in VP2 mRNA transcripts at 3 days rose further to peak in the gut after 5 days of vaccination. The second segment of the gut (as described by Rombout et al. [53]) is considered the region where a strong uptake of macromolecules takes place after the fish were fed for 3 days. However, while VP2 expression peaked in the kidney at 15 days pv, only basal levels of expression were recorded in the gut at that time of vaccination, suggesting that plasmid DNA was then redistributed to other tissues and only small amounts of VP2 expression persisted at this site.

The pattern of VP2 expression was different in the gut and kidney, peaking at 5 and 15 days pv, respectively, and producing stronger expression in the intestine. This is interesting because our knowledge of mucosal immunity is progressing and the recent discoveries regarding gut immunoglobulin open up new opportunities for the study of oral vaccines. It is believed that the teleost fish intestine does not contain organized lymphoid tissue but rather, the equivalent of M-cells that can capture, transport and present antigens to the underlying mucosal immune system [53,54]. The distal intestine of salmonids is well known to have strong vesicular transport activity compared to the proximal intestine [55]. The passage of an oral DNA vaccine through the gastrointestinal tract may be crucial because it is believed that intact antigen must reach the distal part of the gut to ensure uptake and transport by the epithelial cells. The strong responsiveness of the gut needs to be paid more attention as there is evidence that the gastrointestinal tract is an entry route for IPNV in Atlantic salmon [54], and that the virus can modulate the barrier function and transport activities of the intestinal epithelium.

The need for new insights into fish vaccination is evidence of the advance in our understanding of innate and adaptive immune responses. A recent review on IPNV vaccines [56] clearly shows that antigen delivery systems and routes of vaccine delivery influence the type of adaptive immune response generated in the host. Oral vaccines against IPNV and other viruses should be explored not only for their systemic immunity but also for the mucosal immunity they can induce. Determining the relevance of the gut as an immune-competent organ will be a promising area of study. Finally, our results indicate that as well as injection, incorporating the vaccine into food pellets seems to be a promising oral delivery method for the vaccination of cultured fish.

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