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# Development of a live vector vaccine against infectious hematopoietic necrosis virus in rainbow trout

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ABSTRACT

Infectious hematopoietic necrosis virus (IHNV) leads to serious disease and economic losses in the salmonid aquaculture industry. The present study aimed to develop an effective and efficient vaccine to protect rainbow trout (*Oncorhynchus mykiss*) against IHNV infection. Administered via the immersion route, a live vector vaccine containing the regions of the IHNV glycoprotein (G) induced immune responses in rainbow trout. Use of the immersion route induced more-efficient mucosal immunity than intramuscular injection vaccination. IHNV G gene expression was detected in the spleens of rainbow trout at 3, 7 and 15 days post-vaccination (dpv). The G gene expression continuously decreased between 3 and 15 dpv. In addition, the expression of TLR-3, TLR-7 and TLR-8 was upregulated after vaccination, and the highest expression levels of IFN-1, Mx-1, Mx-3, Vig-1 and Vig-2 were observed at 3 dpv. Four markers of the adaptive immune response (CD4, CD8, IgM and IgT) gradually increased. When experimental fish were challenged with IHNV by immersion, significant differences in cumulative percentage mortality were observed in the vaccinated fish and the unvaccinated (empty-plasmid-vaccinated) fish. The relative survival rate was 92% and 6% in the vaccinated group and empty-plasmid group, respectively. Serum antibody levels gradually increased in the vaccinated fish, unlike in the unvaccinated fish, after 7 dpv. Our results suggest there was a significant increase in fish immune responses and resistance to infection with IHNV following administration of the live vector vaccine. Therefore, this live vector vaccine is a promising vaccine that may be utilized to protect rainbow trout against IHNV.

## 1. Introduction

Infectious hematopoietic necrosis virus (IHNV) causes infectious hematopoietic necrosis (IHN) in salmonids, a disease notifiable to the World Organization for Animal Health and one that leads to great economic losses in cultured salmonid farming [1–4]. The disease has a notably wide distribution and a high infection rate, causing mortalities of up to 90%. IHN outbreaks have been reported in the cultured trout industry in Asia, North America [3,5,6], and Europe [7]. IHN was found in Liaoning Province in 1990, and has since threatened salmonid farming in China owing to the high mortality rate [5].

IHNV is a negative-strand RNA virus belonging to the genus *Rhabdovirus* [2]. IHNV encodes five structural proteins (surface glycoprotein, nucleocapsid protein, phosphorylated protein, matrix protein and polymerase protein) and one non-structural protein, with the following arrangement: 3′–N–P–M–G–NV–L–5' [8]. The surface glycoprotein gene consists of approximately 1530 nucleotides that encode a polypeptide of 508 amino acid (aa) residues; the signal peptide is composed of aa residues 1–20 at the N-terminus, 21–459 represents the ectodomain, 460–482 the transmembrane domain, and 483–508 the endodomain [8,9]. The surface glycoprotein can induce the production of neutralizing antibodies, which are nominally used to study IHNV DNA vaccine development [10–13]. As the practice of raising rainbow trout as a food product becomes more prevalent, in-depth research into the prevention and control of IHNV gains greater significance.

Adomako et al. [14] developed a DNA vaccine that is effective in protecting rainbow trout against IHNV infection. There was no difference in rainbow trout survival between the challenged group and the

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control group at 10 weeks. However, intramuscular injection is required, which makes the DNA vaccine impractical for use in the freshwater rainbow trout farming industry. Zhao et al. [4] designed an oral yeast vaccine against IHN. The relative percentage survival (RPS) was 45.8% in the IHNV-challenged group as compared with 2% in the control group; nevertheless, that vaccine still requires improvement to provide greater protection. Therefore, it is essential to develop more efficient and convenient vaccines for use in salmonid fish farming. For instance, Ballesteros et al. [15] tested whether the DNA vaccine could be effective when encapsulated in alginate microspheres and delivered orally to rainbow trout; RPS was 56% after oral administration of 100 mg of pIRF1A-G encapsulated in alginate microparticles, and 70% when trout were intramuscularly injected with 5 mg of pIRF1A-G vaccine. In addition, Zhao et al. [4] designed an oral yeast vaccine against IHN; RPS was 45.8% in an IHNV-challenged group and 2% in a control group. Xu et al. [16] designed a DNA vaccine constructed with the glycoprotein (G) gene of IHNV. The efficacy of the vaccine was evaluated via expression of the Mx-1 gene in muscle and by measuring the titers of neutralizing antibodies produced in the anterior kidney.

Recombinant adenovirus vectors have been used to transfer exogenous genes into a variety of mammalian cell lines to produce target proteins efficiently [17,18]. The target proteins of recombinant adenovirus vectors can stimulate the body to produce a strong humoral and cellular immune response [19]. Studies have reported that surface glycoproteins can induce the production of neutralizing antibodies and stimulate cellular immunity [20]. The mucosae of teleosts act as physical barriers separating the fish from the external environment and are active immunological sites [21]. The adenovirus vector vaccine can induce more-efficient mucosal immunity than can parenteral immunization, and the adenovirus vector was designed specifically to be administered by immersion to induce an immune response to IHNV [22,23]. Thus, in the present study, we optimized a recombinant adenovirus to express the G gene in HEK-293 cells to obtain high titers for the production of a live vaccine vector.

The current study tested whether the live vector vaccine could be effective when used to immunize rainbow trout via the immersion route. For this purpose, the spleens of vaccinated fish were examined to detect expression of the IHNV G gene and several innate immune and adaptive immune markers of the immune responses, including IFN-1 [24,25], Mx-1 [26], Mx-3 [26], Vig-1 [27], Vig-2 [28], TLR-3 [29,30], TLR-7 [30], TLR-8 [30], IgM [31,32], IgT [33], CD4 and CD8 [34]. In addition, we evaluated the efficacy of the live vector vaccine in inducing anti-IHNV antibodies and protecting against IHNV infection in rainbow trout.

## 2. Materials and methods

### 2.1. Ethical statement

All procedures were performed in strict accordance with the recommendations of the Chinese standard 'Laboratory Animal – Guideline for Ethical Review of Animal Welfare' (GB/T 35892–2018). All experiments were approved by the Office of Animal Management of the College of Veterinary Medicine at Gansu Agricultural University.

### 2.2. Plasmid, bacterial strains and reagents

Pad-Track-CMV plasmid, pAd-easy-1 plasmid, and BJ5183 *Escherichia coli* competent cells were purchased from Wuhan Jin Kairui Biological Engineering Co. Ltd (Wuhan, Jiangsu, China). BJ5183 *E. coli* was used as a bacterial host in the experiment. A Quick-Fusion Cloning Kit, RNA extraction kit, DNA extraction kit, plasmid extraction kit, DNA polymerase and restriction endonuclease were purchased from New England Biolabs (Ipswich, MA, USA). Rabbit anti-IgG (rabbit anti-infectious hematopoietic necrosis virus polyclonal antibody IgG fraction obtained by immunizing rabbits) was purchased from Creative Diagnostics (New York). Lipofectamine™ 3000 Reagent was purchased from Invitrogen (Carlsbad, CA, USA), and chemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

All primers were designed from sequences sourced from NCBI databases. Amplification of DNA, extraction of plasmid, and molecular operations were performed in accordance with instructions for the reagent kits. The specificity of all primers used in the study were tested using NCBI Primer-Blast.

### 2.3. Amplification of the G gene open reading frame (ORF)

After reverse transcription, cDNA samples were amplified for the target gene using G gene primers. The pair of cloning primers were a forward primer 5′-ATCTCTAGACCATGCGTCGACGCCACCATGTACA CCATGATCACC-3′ and a reverse primer 5′-TAGATCTTCGAATCCCTCG AGTTAGGACCGGTTT GCCAGGTGATA-3′ designed from sequences acquired from NCBI's GenBank.

Real-time PCR amplification was performed in a 50-μl reaction volume containing 1 × PrimeSTAR GXL Buffer, dNTP mixture at a concentration of 200 μM, 2.5 units of PrimeSTAR GXL DNA Polymerase, 2 μl of cDNA, and primers at a concentration of 0.2–0.3 μM; water was added to bring the total volume to 50 μl. Amplification was performed in a thermal cycler (Bio-Rad; Hercules, CA, USA) for 31 cycles. The amplification parameters were: initial denaturation at 94 °C for 2 min, 10 s of denaturation at 98 °C, 30 s of annealing at +60 °C, 1 min of extension at 72 °C, and a final extension for 10 min at 72 °C. 10 μl of products were electrophoresed on 1.5% agarose gel (Sigma-Aldrich) and then photographed under ultraviolet light. Sequence determination was performed after identifying positive products, which were BLASTed against the target sequences.

### 2.4. Quick fusion of G gene and pAd-track-CMV vector

A Quick-Fusion Cloning Kit was used to set up reaction mixtures containing 40 ng of positive PCR products, 20 ng of linearized pAd-Track-CMV, 1 μl of fusion enzyme, 2 μl of 10 × fusionase buffer, and water to make a total volume of 20 μl. The reactions were allowed to incubate for 30 min at 37 °C. Next, 10 μl of fusion product was immediately transformed into 40 μl of competent cells. The competent cells were incubated for 30 min on ice, then placed in a water bath at 42 °C for 30 s, and immediately resurrected at 37 °C for 1 h. The cells were plated in Petri dishes with 50 μg/ml kanamycin. The cells were grown for 16–20 h at 37 °C; afterward, 15–20 colonies were picked and grown in 5 ml of L-broth with 50 μg/ml of kanamycin. The plasmid was extracted according to the kit manufacturer's instructions (NEB) and identified by PCR amplification. The PCR reaction conditions were the same as used for the amplification of samples. PCR products were run on 1.5% agarose gel, and the gels were photographed under ultraviolent light.

The PCR products were digested using *Sal*I and *Xho*I restriction enzymes. Digestion reactions included 1 μg of DNA, 1 μl of restriction enzyme, 5 μl of 10 × buffer, and water to create a final volume of 50 μl. The reactions were maintained at 37 °C for 2 h, and the fragments were separated on an agarose gel, and the gel was photographed under ultraviolet light [35]. The positive products were identified and sequenced. BLAST sequence analysis was used to identify amino-acid differences between the PCR products and the target sequences. The pAd-Track-CMV vector plasmids were used as a negative control.

### 2.5. Homologous recombination of recombinant adenovirus and pAd-Easy-1 vector

Linearized recombinant vector plasmids were extracted for digestion with *Pme*I (NEB). The digestion was performed in 10 μl of 10 × buffer, 1 μl of *Pme*I enzyme, and 1.0 μg of recombinant vector plasmid. The digestion products were purified by phenol/chloroform

extraction and ethanol precipitation, according to standard protocols. 10 μl of the linearized recombinant vector plasmid was transformed into 40 μl of electro-competent BJ5183 cells in an electroporator with a 2.0-mm electric rotor at 2500 V, 200 Ω, and 25 μF, for 5 ms. Competent cells were resurrected at 37 °C for 1 h. Next, electroporated cells were seeded on Petri dishes with L-agar supplemented with 50 μg/ml kanamycin. The smaller colonies were picked and grown at 37 °C for 16–20 h in 5 ml of L-broth containing 50 μg/ml of kanamycin, and thereafter grown on L-agar for an additional 16–20 h [35].

Recombinant adenoviral plasmids were identified on 0.8% agarose gel and photographed under ultraviolet light. The plasmids were tested by digestion with *Pac*I (NEB) at 37 °C for 4 h. The digestion reaction contained 1 μg of positive adenoviral plasmids, 5 μl of 10 × buffer, 1 μl of restriction endonuclease *Pac*I, and water to create a total volume of 50 μl.

Positive recombinant adenoviral plasmids were transformed into competent *E. coli DH10B* cells using the same conditions as the previous electroporation. Next, recombinant adenoviral plasmids were extracted from *E. coli DH10B* and digested by *Pac*I enzyme [35]. The DNA products were extracted for use in transfection. The recombination empty plasmids of pAd-Track-CMV empty vector and pAd-Easy-1 vector were used as a negative control.

### 2.6. Transfection of recombinant adenoviruses into HEK-293 cells

HEK-293 cells were seeded at $1.0 \times 10^6$ mL$^{-1}$ in 25-cm$^2$ flasks and cultured for 24 h before transfection. At the time of transfection, the cells were approximately 50–70% confluent. 20 μl of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was added to 480 μl of DMEM to make culture suspensions. Linearized recombinant DNA (4 μg), added to the culture suspensions at room temperature for 20 min, was transfected into the cells. After transfection, the cells were monitored for the expression of green fluorescence protein (GFP), for 2–8 days, and finally harvested. After being thawed twice, the viruses were used to inoculate cells in 25-cm$^2$ flasks. The virus was harvested for three days, according to GFP expression. HEK-293 cells transfected with recombination empty plasmid were used as a negative control [35].

### 2.7. Identification of antigen with western blotting

The infected HEK-293 cells were collected and lysed via sonication (40 W for 3 s, pause for 3 s, repeated for 20 cycles). The proteins were extracted from the lysate using RIPA buffer (5 min). The samples were centrifuged at 12 000 rpm for 5 min at 4 °C, and the supernatant was transferred to a new tube. The infected HEK-293 cells were collected, lysed with RIPA lysis buffer (5 min), and the proteins were extracted. The samples were collected and centrifuged, at 12 000 rpm for 5 min at 4 °C, after ultrasonic disruption (i.e. 40 W for 3s, paused for 3 s, and repeated 20 times). The supernatant was prepared by addition of protein loading buffer at 95 °C for 10 min. The protein samples were electrophoresed on SDS-PAGE gels at 60 V for 60 min, and held at 120 V until the end; β-actin was used as a loading control. The proteins were electrotransferred to a PVDF membrane (200 mA for 120 min). The membrane was blocked for 1 h with bovine serum albumin (50 g/L) and then washed three times with PBST buffer (10 min per wash). The membrane was incubated, with shaking, with rabbit anti-IgG diluted at an appropriate dilution, for 1 h, and washed three times with PBST buffer (10 min per wash). The membrane was then incubated with anti-rabbit secondary antibody for an appropriate duration of 2 h and again washed three times with PBST buffer. The developing reagent was added to initiate the chemiluminescent reaction for detection [36].

### 2.8. Determination of recombinant adenovirus titers

The viruses were diluted continuously with DMEM from $10^{-1}$ to $10^{-10}$ in ten-fold series. The diluted viruses were plated into 96-well plates containing HEK-293 cells; 100 μl of each dilution was inoculated into 8 wells. The wells were observed daily and the results recorded for 2–8 days. Results were calculated using the Reed and Muench method [37].

### 2.9. Fish rearing conditions

Healthy rainbow trout specimens (mean weight 3–4 g), with no history of viral diseases, were purchased from a springwater farm in Yongdeng County, Gansu Province, China. The fish were maintained at the Center for Fishery Technology Promotion in Lanzhou, in a flowing-water 400-L pool, at 14 ± 1 °C, and fed daily (2.0% body weight) with 4-mm-diameter pelleted food (Ruixing Biotechnology Co. Ltd, China). The fish were acclimated to the laboratory conditions for two weeks before the experimental procedures began, and no clinical signs were observed. Furthermore, no IHNV or other salmonid viruses were detected through testing with carp epithelial (EPC) cells. The EPC cells were acquired from the Shenzhen Center of Technology of Customs Food Inspection and Quarantine, and were grown at 18 °C for 48 h.

### 2.10. Safety assessment of the live vector vaccine

For the immunization trials, the fish were divided into three groups of 30 fish each; the weights of the fish ranged from 3 to 4 g. Fish of group 1 were administered a 1:10 dilution of the titer with $1 \times 10^{10.5}$ mL$^{-1}$ $TCID_{50}$ of the live vector vaccine, via 10-min immersion. Fish of group 2 were administered 0.1 ml of the live vector vaccine via injection. Group 3 was a control group comprising unvaccinated fish. Dead fish were collected daily for seven days post-vaccination. To assess the safety of the live vector vaccine, the two vaccinated groups were observed for clinical adverse effects for seven days [38]. The lengths and weights of the fish in these two groups were recorded for 60 dpv.

### 2.11. Immunization trials

For the immunization trials, the fish were divided into three groups of 150 fish each; the weights of the fish ranged from 3 to 4 g. Fish in group 1 were administered a 1:100 dilution of the titer with $1 \times 10^{10.5}$ mL$^{-1}$ $TCID_{50}$ of the live vector vaccine, via 10-min immersion. Fish in group 2 were administered a 1:100 dilution of the titer with $1 \times 10^{10.5}$ mL$^{-1}$ $TCID_{50}$ of the empty plasmid, via 10-min immersion. Group 3 was a control group comprising unvaccinated fish.

### 2.12. Sample and cell lines

Total RNA was extracted using a Qiagen RNA extraction kit (Qiagen Inc., Germantown, MD, USA), according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA using a Roche Transcriptor First Strand cDNA Synthesis Kit, in accordance with the manufacturer's instructions. The reaction was prepared with 1 μg of total RNA, 2.5 μM of Anchored Oligo(dT)18 Primer, 10 U/μL 20 U Transcriptor Reverse Transcriptase, 1 × Transcriptor Reverse Transcriptase reaction buffer, 20 U/μL 20 U Protector RNase Inhibitor, and 1 mM deoxynucleotide. The reaction volume was brought to 20 μl with water. The reaction of cDNA synthesis was carried out in a Bio-Rad T100 Thermal Cycler (Hercules, CA, USA) with a heated lid, for 10 min at 65 °C and 30 min at 55 °C, according to the manufacturer's instructions.

Human embryonic kidney cells (HEK-293) were acquired from the Lanzhou Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. Cells were cultured in DMEM F-12 with 10% fetal bovine serum (FBS) (Biological Industries). The cells were grown at 37 °C for 24 h in 5% $CO_2$ and 95% humidity.

### 2.13. Quantification of gene expression by two-step quantitative reverse-transcription PCR (qRT-PCR)

A total of 4 fish were sacrificed via MS-222 (Sigma-Aldrich, Louis, MO, USA) overdose at each time point [15]. Spleens were aseptically harvested from vaccinated and unvaccinated rainbow trout and stored individually in 1 mL of TRIzol Reagent (Invitrogen, Spain) at −80 °C. The samples were homogenized in an Absolute 1100 High-Efficiency Tissue Crusher (Monad Biotechnology, Suzhou, China), for 3 min at 50 Hz, with broken beads. Total RNA was extracted from the different tissues using the TRIzol Reagent according to the manufacturer's instructions. The purity and yield of the RNA samples were analyzed using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The quality of RNA was determined by measuring the ratio of absorbance at 260/280 nm, and a 1.8:1.9 ratio was considered acceptable for RNA purity. Total RNA was reversely transcribed to cDNA using a Roche reverse transcriptase kit, according to the manufacturer's protocol. The 20-μl reaction volume contained 1 μg of total RNA, 2.5 μM of Anchored Oligo(dT)18 Primer, 10 U/μL of reverse transcriptase, 1 × reverse transcriptase buffer, 20 U/μL protease inhibitor, 1 mM deoxynucleotide mixture, and RNase-free water. The reaction conditions were: 65 °C for 10 min, 30 min at 55 °C, and 85 °C for 5 min. The 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 total volume containing 12.5 μL of SYBR Green (Life Technologies, Carlsbad, CA, USA), 0.5 μM each of the forward and reverse primers, 8.5 μL RNase-free water, and 2 μL of cDNA. The amplification of cDNA was accomplished in a real-time PCR detection system (Bio-Rad Laboratories, Spain) under the following conditions: 1 cycle of 94 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and a dissociation cycle (1 min at 95 °C, and 1 min at 60 °C). The melting curve of each amplicon was analyzed to determine the specificity of the amplification. Table 1 lists the accession numbers and the corresponding primer sequences of the amplified rainbow trout genes [15].

The trout elongation factor 1-alpha (EF-1α) was used as the endogenous gene control to normalize the expression of each gene in the RNA samples. The rainbow trout β-actin gene was used as the endogenous control gene for the normalization of PCR amplifications of the expression of the G gene for IHNV. The results were analyzed using optical system software (Bio-Rad) and expressed as 2-ΔCT, where ΔCT is determined by subtracting the threshold cycle (CT) of EF-1α (β-actin) from the CT of the identified genes. PCR amplification was performed at least three times, and each experiment was repeated three times to confirm the results.

### 2.14. IHNV experimental challenge by immersion

At 15 dpv, 50 fish (average weight 3 g) from each experimental group were challenged by immersion in a bath of $10^5$ $TCID_{50}$ $mL^{-1}$ of IHNV for 3 h at 14 ± 1 °C [15]. Dead fish were collected daily for 30 days post-challenge. Moribund fish displayed the typical signs of IHNV infection, such as a darkened body and a distended abdomen. The dead fish were assessed by PCR amplification of the IHNV N gene to confirm death as a result of IHNV infection. Efficacy of the live vector vaccine was assessed by comparing the values of total cumulative percent mortality (CPM) among the three experimental fish groups. Relative percentage survival (RPS) of each experimental group was calculated according to the formula: RPS = [1 − (CPM vaccinated fish/unvaccinated fish)] × 100 [15].

### 2.15. Neutralization trial for the detection of anti-IHNV serum antibodies

At 7, 14, 21, 28 and 35 dpv and without IHNV challenge, 10 fish were sacrificed at each time point by immersion in an overdose solution of MS-222. Blood was collected from the caudal vein of experimental fish at each time point. The blood samples were set aside and allowed to clot at 37 °C for 2 h, stored at 4 °C overnight, and then centrifuged (2500 *g*) at 4 °C for 10 min. Serum samples were collected and stored at −20 °C until they were analyzed by neutralization trial. The $TCID_{50}$ of IHNV was determined in EPC cells.

The serum samples were inactivated at 65 °C for 30 min. The inactivated serum was diluted with DMEM into 10 different dilution factors (neat, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512) mixed with 100 $TCID_{50}$ of IHNV, and neutralized at 37 °C for 60 min; 0.1 mL of neutralizing solution including inactivated serum and virus at different concentrations was added to the wells of 96-well plates containing confluent EPC cells. Positive and negative control groups were included. The plates were incubated at 37 °C for 24 h in an atmosphere containing 5% $CO_2$. While the positive hole exhibited a 100% cytopathic effect (CPE), the serum hole was judged negative if it demonstrated 100% CPE, or positive if it demonstrated less than 50% CPE. The neutralization index (NI) was calculated according to the Karber method: NI = test group $TCID_{50}$/control group $TCID_{50}$ [39].

After analyzing the results, the serum was judged to contain neutralizing antibodies if the NI value was > 50. If the NI value was 10–50, the serum was judged to be indeterminate; in cases where NI was < 10, the serum was judged to contain no antibodies.

### 2.16. Statistical analysis

Differences in CPM between the groups of experimental and control fish were analyzed statistically, and Fisher's exact precise test was applied using GraphPad Prism version 5.0. Differences in gene expression between each group were analyzed via factorial ANOVA. The control group was used to determine the statistical significance of gene-expression levels gained from all the immunized experiments. In all analyses, $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Analysis of recombinant plasmid

The length of the G gene was 1533 bp, as anticipated (Fig. 1A). The results showed that the G gene recombination plasmid was successfully constructed and this was confirmed by enzyme digestion with *Sal*I and *Xho*I (Fig. 1B).

### 3.2. Identification of homologous recombination

The recombinant adenovirus plasmid refers to the recombination of pAdTrack-CMV and pAdEasy-1. The recombinant adenovirus plasmid was confirmed by PCR and digestion with *Pac*I. The length of the G gene product was 1533 bp. After digestion, we obtained two clear bands of about 33 kb and 4.5 kb (Fig. 1C). Therefore, the recombinant adenovirus plasmid was successfully constructed.

### 3.3. Identification of antigen

The recombinant adenovirus was transfected into HEK-293 cells. Western-blot analysis was used to detect expression of glycoproteins, and β-actin was used as an internal control. The results indicated that, compared with the empty-vector and blank HEK-293 cells, the recombinant adenovirus had a band of protein at a molecular mass of about 58 kD (Fig. 1D). Thus, glycoprotein expression was confirmed in the recombinant adenovirus.

### 3.4. Detection of recombinant adenoviruses titers

GFP appeared in 80% of the HEK-293 cells after transfection with the recombinant adenovirus (Fig. 1E). The titer of recombinant adenovirus was $1 \times 10^{10.5}$ $mL^{-1}$ $TCID_{50}$.

**Table 1**
Accession numbers of the amplified genes of rainbow trout and the primer sequences used in the qRT-PCR assays.

| Code | Name | Abbreviation | Primer sequence (5′–3′) | Size |
|---|---|---|---|---|
| NM_001124531 | Type I interferon | IF-1 | AAAACTGTTTGATGGGAATAT | 228 bp |
| | | IR-1 | CGTTTCAGTCTCCTCTCAGGT | |
| NM_001171901 | Interferon-induced protein Mx1 | MF-1 | AGCTCAAACGCCTGATGAAG | 142 bp |
| | | MR-1 | ACCCCACTGAAACACACCTG | |
| U47946.1 | Interferon-induced protein Mx3 | MF-3 | AGCTCAAACGCCTGATGAAG | 138 bp |
| | | MR-3 | TGAATATGTCTGTTATCCTCC | |
| AF076620.1 | Viperin 1 | VF-1 | ACGACCTCCAGCTCCCAAGT | 173 bp |
| | | VR-1 | GTCCAGGTGGCTCTTCCTGC | |
| AF291718.1 | Viperin 2 | VF-2 | CCACCCACGTCATATCAGGG | 145 bp |
| | | VR-2 | AACGCAGACGCTTGTTGGC | |
| ABE69177 | Toll-like receptor 3-like protein | TLRF-3 | AGCCCTTTGCTGCCTTACAG | 207 bp |
| | | TLRR-3 | TTGTTGATTCGACAGGTGG | |
| GQ422119 | Toll-like receptor 7 | TLRF-7 | TACAGCTTGGTAACATGACTC | 88 bp |
| | | TLRR-7 | CAACTCTCTGAGACTTGTCGG | |
| GQ422120 | Toll-like receptor 8a2 | TLRF-8 | CAGCATTGAACGGGACAG | 90 bp |
| | | TLRR-8 | CATTGTCATAGGCCAGGT | |
| AY973030.1 | T-cell surface glycoprotein CD4 | CDF-4 | CCTGCTCATCCACAGCCT | 111 bp |
| | | CDR-4 | CTTCTCCTGGCTGTCTGA | |
| NM_001124263 | T-cell surface glycoprotein CD8 alpha precursor | CDF-8 | AGTCGTGCAAAGTGGGA GGTTGCAATGGCATACAG | 123 bp |
| | | CDR-8 | | |
| X65263.1 | Membrane-bound immunoglobulin M | IgMF-1 | ACCTTAACCAGCCGAAAG TGTCCCATTGCTCCAGTC | 78 bp |
| | | IgMR-1 | | |
| AY870265 | Immunoglobulin Tau heavy chain | IgTF-1 | AGCACCAGGGTGAAACC GCGGTGGGTTCAGAGTC | 72 bp |
| | | IgTR-1 | | |
| AF498320 | Elongation factor 1 (EF1)-alpha | EFF-1 | GATCCAGAAGGAGGTCA | 150 bp |
| | | EFR-1 | TTACGTTCGACCTTCCAT | |
| L40883 | Nucleocapsid protein | NF-1 | TGTGCATGAAGTCAGTGGT | 107 bp |
| | | NR-1 | CCTGCTCATCATGACACCG | |
| U50401.1 | Glycoprotein protein | GF-1 | GCGCACGCCGAGATAATATCAA | 121 bp |
| | | GR-1 | TCCCGTGATAGATGGAGCCTTT | |
| | | GP-1 | CGATCTCCACATCCCGGAATAAATGACGTCT | |
| AJ438158 | β-actin | β-actin-F | GGCCGTGTTGTCCCTGTAC | 48 bp |
| | | β-actin-R | CCGGAGTCCATGACGATACC | |
| | | β-actin-P | CCTCTGGCCGTACCACC | |

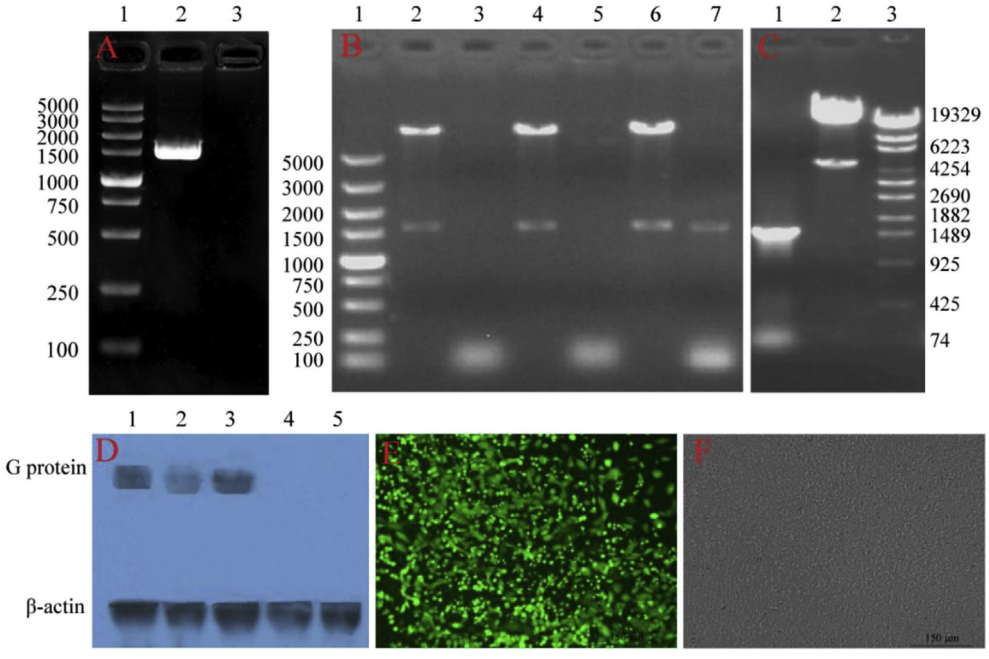


**Fig. 1. Construction of recombinant adenovirus.** A) PCR amplification of the infectious hematopoietic necrosis virus (IHNV) G gene. Lane 1: 5000 bp DNA marker. Lane 2: G gene product. B) PCR amplification and enzymatic digestion of recombinant plasmids. Lane 1: 5000 bp DNA marker. Lanes 2, 4, 6: recombinant plasmids digested by *Sal*I and *Xho*I. Lanes 3, 5: PCR amplification of the empty vector. Lane 7: PCR amplification of recombinant plasmid. C) Screening of recombinant adenovirus vector. Lane 1: PCR amplification of the G gene. Lane 2: recombinant plasmids digested by *Pac*I. Lane 3: 5000 bp DNA marker. D) Western-blot analysis of the recombinant adenovirus G protein expression. Lanes 1, 2, 3: G gene expression of the recombinant adenovirus harvested from HEK-293. Lane 4: recombination empty plasmid harvested from HEK-293. Lane 5: HEK-293 cells with the control. E) Green fluorescence protein (GFP) expression in HEK-293 cells infected with recombinant adenovirus, after 48 h. F) HEK-293 cells with the control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

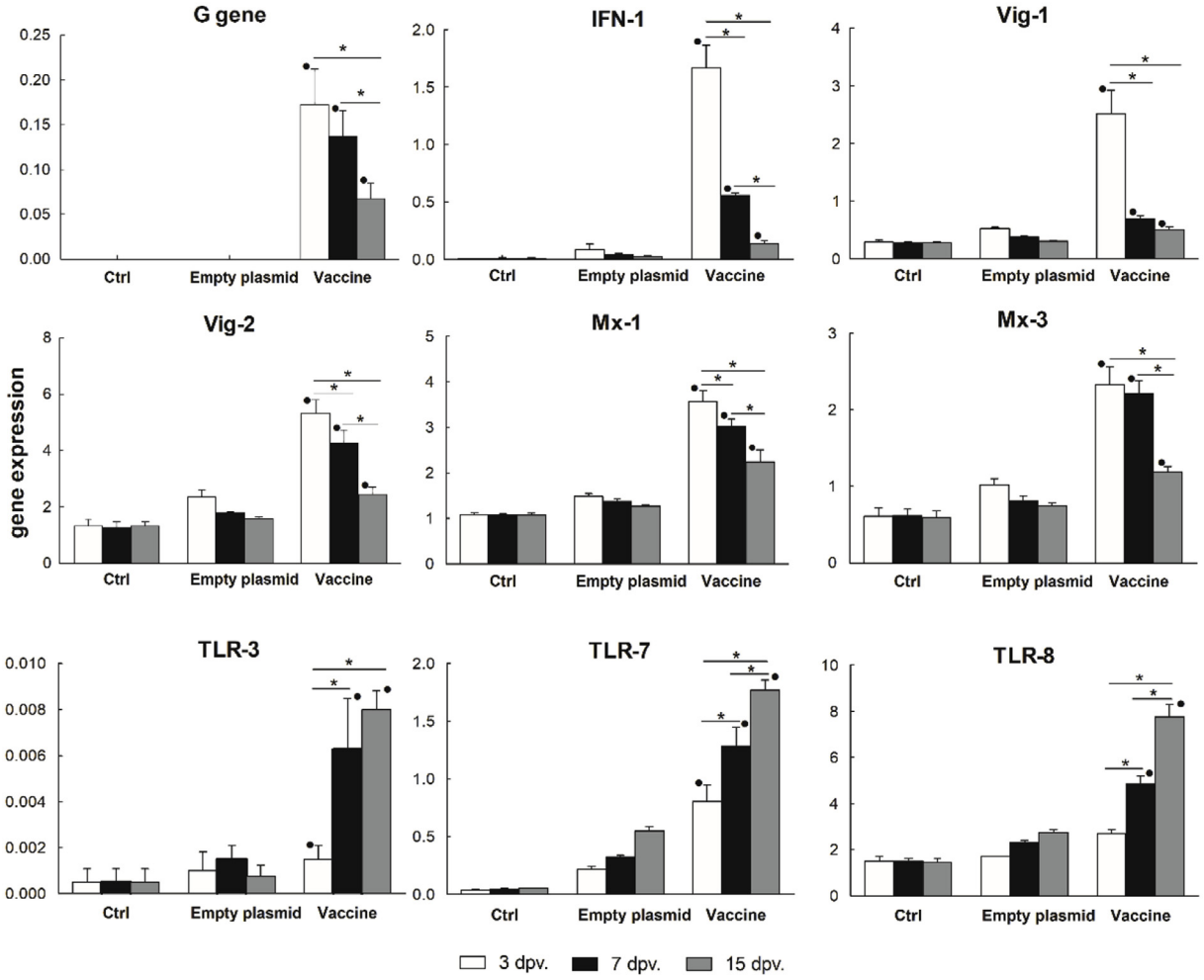


**Fig. 2. IHNV-G, TLRs, IFN-1, and IFN-inducible gene expression in the spleens of rainbow trout after immunization, for the different experimental groups.** Different groups of fish were immunized with the live vector vaccine and the empty plasmid. IFN-1, Mx-1, Mx-3, Vig-1, Vig-2, TLR-3, TLR-7 and TLR-8 gene expression in the spleens of four vaccinated fish were evaluated by qRT-PCR at 3, 7 and 15 days post-vaccination (dpv). The expression of the IHNV G gene was also used as a vaccine marker. Unvaccinated fish were used as negative controls. The results were analyzed using optical system software (Bio-Rad) and expressed as $2^{-\Delta CT}$, where ΔCT was determined by subtracting the threshold cycle (CT) of EF-1α (β-actin) from the determined CT of the genes. Asterisks (*) indicate significant differences in the levels of gene expression ($P < 0.05$). Black dots indicate significant differences between the vaccinated and unvaccinated groups of fish.

### 3.5. Clinical safety assessment of the live vector vaccine

Dead fish were not found in the two vaccinated groups for 7 dpv and no adverse reactions were displayed by these fish within seven days; this proved the safety of the live vector vaccine. The lengths and weights of the fish did not differ significantly between the experimental and control groups at 60 dpv.

### 3.6. IHNV-G, TLRs, IFN-1, IFN-inducible genes, CD4, CD8, IgM and IgT gene expression in response to the vaccine

Spleens were collected to detect the expression of IFN, Mx-1, Mx-3, Vig-1, Vig-2, TLR-3, TLR-7 and TLR-8 using qRT-PCR. EF-1α was used as an internal control. The IHNV G gene was one of the items tested for gene expression in rainbow trout immunized with the recombinant virus. As shown in Fig. 2, the expression of IFN-1, viperin-1, viperin-2, Mx-1, Mx-3 and IHNV G genes was highest at 3 dpv, and decreased over the period 3 to 15 dpv. The expression level of TLR-3, TLR-7 and TLR-8 continuously increased from 3 dpv to 15 dpv, and the highest expression levels were measured at 15 dpv.

The rainbow trout CD4, CD8, IgM, and IgT genes were selected for analysis of the cellular-specific immune responses induced by the administration of the live vector vaccine. The unvaccinated group was used as the gene-expression level of statistical significance. As shown in Fig. 3, the expression level of CD4 and CD8 genes in the experimental animals was significantly increased from 3 dpv to 15 dpv. CD4 expression levels were higher than those of CD8 in both the experimental and control groups. A significant increase in IgM and IgT expression was detected in the spleens of the vaccinated trout.

### 3.7. Vaccine protected fish against a lethal challenge of IHNV

Mortality was recorded daily for 30 days after the trout were challenged with IHNV by immersion. As shown in Fig. 4, there were fewer mortalities among vaccinated fish than among unvaccinated fish. CPM was 8% in fish immunized with the vaccine, and 100% in unvaccinated fish. Additionally, RPS was 92% and 6% for the vaccinated and the empty-plasmid fish, respectively.

### 3.8. Detection of anti-IHNV serum antibodies

In the present study, samples of the pre-immune blood were collected as a blank control from both the control and experimental groups. As shown in Fig. 5, the neutralizing antibody levels of the vaccinated group were increased and positive (NI > 50) at 7 dpv. However, the empty-plasmid group and the unvaccinated group

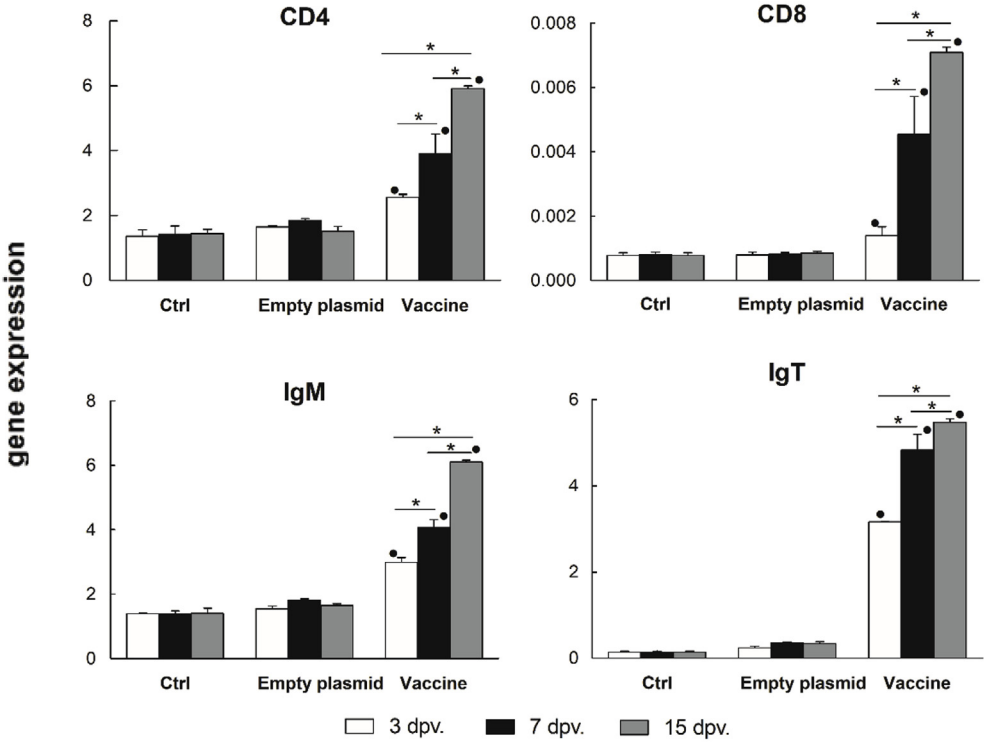


**Fig. 3. CD4, CD8, IgM and IgT gene expression in the spleens of rainbow trout after immunization, for the different experimental groups.** Different groups of fish were immunized with the live vector vaccine and the empty plasmid. CD4, CD8, IgM and IgT gene expression in the spleens of four vaccinated fish were evaluated by qRT-PCR at 3, 7 and 15 days post-vaccination (dpv). Unvaccinated fish were used as negative control. The results were analyzed using optical system software (Bio-Rad) and expressed as $2^{-\Delta CT}$, where ΔCT was determined by subtracting the threshold cycle (CT) of EF-1α (β-actin) from the determined CT of the genes. Asterisks (*) indicate significant differences in the levels of gene expression ($P < 0.05$). Black dots indicate significant differences between the vaccinated and unvaccinated groups of fish.

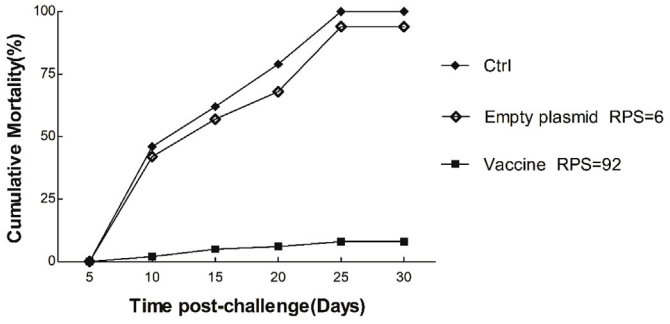


**Fig. 4. Cumulative percent mortality (CPM) of rainbow trout after vaccination and subsequent challenge with IHNV.** A group of 150 fish were treated with the live vector vaccine via the immersion route. The control group comprised unvaccinated fish and the empty-plasmid vaccinated group. At 15 days post-vaccination, 50 fish from each experimental group were challenged with IHNV ($10^5$ $TCID_{50}$ $mL^{-1}$) by immersion, and then monitored for 30 days. The CPM of each experimental group was recorded, and the relative percentage survival (RPS) was calculated using the formula RPS = [1 – (% mortality vaccinated fish/% mortality unvaccinated fish) – 100]).

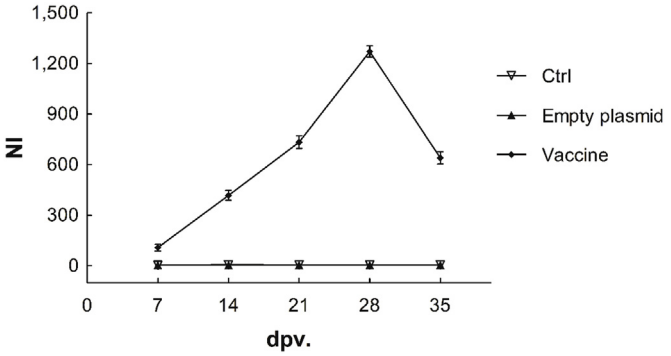


**Fig. 5. Dynamic model of antibody levels after immunization of the rainbow trout.** At 7, 14, 21, 28 and 35 days post-vaccination (dpv) without IHNV challenge, the blood was collected from the caudal vein of 10 experimental fish at each time point. The corresponding serum samples were tested for anti-IHNV antibodies by neutralization trial. The neutralization index (NI) was calculated according to the Karber method: NI = test group $TCID_{50}$/control group $TCID_{50}$. The diagram represents mean antibody levels for the 10 fish tested at each time point.

showed no significant change, and the NI values of serum antibody in the empty-plasmid and the unvaccinated groups were 20–45, much lower than in the vaccinated group (NI > 50).

## 4. Discussion

IHN is a viral disease with a high mortality rate affecting mainly salmonid fish; this disease has caused serious economic losses in the global salmon and trout farming industries in recent years [40,41]. In China, IHN was first discovered in Liaoning Province, in 1990. Since then, the range of the disease has spread, and outbreaks occur frequently due to the lack of effective preventive measures [16].

Vaccination is an important measure to prevent and control outbreaks of this disease. Previously, a DNA vaccine shown to be very effective in protecting fish against IHNV was patented in Canada and approved for commercialization, in July 2005, by the Canadian Food Inspection Agency. However, the insertion of a promoter from a human pathogen makes DNA vaccines containing this promoter 'unsafe' for use in some countries, thus its commercialization is restricted due to safety concerns [15]. Kurath et al. [32] developed a pIHNw-G DNA vaccine against IHNV, with neutralizing antibodies lasting for three months post-immunization in rainbow trout. However, that vaccine must be administered intramuscularly, limiting its practical use in the freshwater rainbow trout farming industry. Previous research has proven that the recombinant adenovirus shows high transfection efficiency and does not integrate into the host genome. It is a potent viral vector that can be used to transfect the target disease genes *in vivo* and *in vitro*. Compared with traditional vaccines, the good immunogenicity and safety of this recombinant adenovirus make it an ideal gene-transfer vector for establishing a vaccine platform [42,43]. In the present study, we constructed a recombinant adenovirus with glycoprotein and

applied the vaccine using the immersion method, which is more convenient than intramuscular challenges, to immunize freshwater rainbow trout.

Fish depend mostly on innate immunity and their specific immune system. The spleen was selected as the target organ for transcriptional analysis because it plays an important role in maintaining normal immune function [44]. IHNV G gene expression was detected by qRT-PCR in the spleens of vaccinated fish. The highest levels of G gene expression were measured at 3 dpv. Continuous decrease of G gene expression from 3 dpv to 15 dpv showed that the live vector vaccine certainly conferred an immune effect on rainbow trout. As expected, our results suggest that the expression of IFN-1 gene was induced at 3 dpv and 7 dpv in the spleens of the vaccinated fish. In addition, Vig-1 and Vig-2 gene expression significantly showed that the external virus protein is sufficient for immune induction [27,28]. In this study, the expression of Vig-1 and Vig-2 genes was significantly up-regulated in the spleens of vaccinated fish as compared with the levels in the spleens of unvaccinated fish. Remarkably, the significant expression of Vig-1 and Vig-2 reached the highest levels at 3 dpv. These results indicate that IHNV G gene expression was able to induce gene expression of Vig-1 and Vig-2. Moreover, some reports have found that Mx proteins from rainbow trout might interfere with IHNV replication [26]. Likewise, our results showed that the live vector vaccine caused a significant induction of Mx-1 and Mx-3 gene expression at 3 dpv in the spleens of vaccinated fish.

TLRs are transmembrane proteins that recognize conserved pathogen structures. Some fish studies have found that expression of TLRs is altered by viral infections [30,45]. Toll-like receptor 3 (TLR3) relates to IFN gene expression in fish cells [29]. In addition, the Toll-like receptors (TLR) 7 and 8 bind single-stranded RNA of viral origin [30]. Our studied demonstrated that the expression of TLR-3, TLR-7 and TLR-8 was affected by the vaccine. Compared with the results for unvaccinated fish, the live vector vaccine induced higher expressions of TLR-3 and TLR-7, and the level of expression continued to rise at 3, 7 and 15 dpv. However, there were significant differences in TLR-8 expression between vaccinated fish and unvaccinated fish at 7 dpv and 15 dpv. From our results we can also infer that Toll-like receptors might act as important mediators of IHNV live vector vaccine in rainbow trout.

In fish, as in higher vertebrates, there is a complex mucosa-associated lymphoid tissue in the intestine, skin, gills and nasopharynx, and these tissues have a specific immune response [46,47]. Studies have reported numerous $CD4^{-}$and CD8-expressing cells were identified in the mucosa [48]. In this study, the live vector vaccine specifically activated the immune cells on the mucosal surfaces. The expression levels of CD8 and CD4 continued to increase from 3 dpv to 15 dpv, and after the 15th day the levels were significantly higher in vaccinated fish than in the controls.

In teleost fishes, IgT is a primitive immunoglobulin specialized for mucosal immunity and it plays an important role in the mucosal immune response [33]. Long-term-memory efficacy of the vaccine was induced by specific stimulation of the mucosa-associated IgT [15]. In addition, the spleens of teleosts contain a large number of B cells, and B cells can produce IgM, which responds to pathogens in the systemic and mucosal compartments [49]. In teleosts, IgM responses are typically characterized as having high titers in response to vaccination [50]. In this study, IgM and IgT gene expression in the spleen of rainbow trout was detected after administration of the live vector vaccine, indicating significant induction of IgM and IgT gene expression in the spleens of vaccinated trout. The levels of expression were significantly different at 3, 7 and 15 dpv. However, no significant differences in IgT and IgM production were detected in the spleens from the empty-plasmid group and the unvaccinated fish. This suggests that the live vector vaccination produced a more-intense immune response than the immunization with empty plasmid or no vaccination.

In the experimental group, the vaccine was administered to rainbow trout and the RPS and CPM were used to evaluate vaccine efficacy in inducing a protective immune response against IHNV challenge. Mortality data were collected daily for 30 days after vaccination. Statistically significant differences in the mortality rate between the experimental and control groups were observed. Administration of the vaccine to rainbow trout protected against viral infection, reaching an RPS value of 92%, whereas RPS was less than 6% in the empty-plasmid group. Differences in RPS between the experimental and empty-plasmid groups might be explained by differences in mortalities in the control and experimental groups. The CPM in the vaccinated and unvaccinated fish was 8% and 100%, respectively. Previous studies have shown that serum antibodies have a neutralizing effect on IHNV [51]. The efficacy of the IHNV vaccine was evaluated by testing the serum antibody levels of vaccinated trout [15,16]. In the current study, the serum antibody levels gradually increased as of 7 dpv, demonstrating that the vaccine provided the rainbow trout with good protection after immunization.

In summary, our results showed that the live vector vaccine is safe for fish vaccination; for best protection, rainbow trout should be immunized with a 1:100 dilution of the titer with $1 \times 10^{10.5}$ $mL^{-1}$ $TCID_{50}$ of the live vector vaccine, via a 10-min immersion route. There was a significant increase in fish immune responses and resistance to IHNV infection, showing that the live vector vaccine was expressed in the spleens of vaccinated trout. The vaccine also stimulated the expression of IFN-1, Mx-1, Mx-3, Vig-1, Vig-2, TLR-3, TLR-7 and TLR-8 genes in the spleens of vaccinated fish. The expression of CD4, CD8, IgM and IgT genes suggested that efficacy of the vaccine is high. Therefore, the live vector vaccine is a promising vaccine with which to protect rainbow trout against IHNV.

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