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A chimeric recombinant infectious hematopoietic necrosis virus induces protective immune responses against infectious hematopoietic necrosis and infectious pancreatic necrosis in rainbow trout

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ABSTRACT

Infectious pancreatic necrosis virus (IPNV) and infectious hematopoietic necrosis virus (IHNV) are two common viral pathogens that cause severe economic losses in all salmonid species in culture, but especially in rainbow trout. Although vaccines against both diseases have been commercialized in some countries, no such vaccines are available for them in China. In this study, a recombinant virus was constructed using the IHNV U genogroup Blk94 virus as a backbone vector to express the antigenic gene, VP2, from IPNV via the reverse genetics system. The resulting recombinant virus (rBlk94-VP2) showed stable biological characteristics as confirmed by virus growth kinetic analyses, pathogenicity analyses, indirect immunofluorescence assays and western blotting. Rainbow trout were immunized with rBlk94-VP2 and then challenged with the IPNV ChRtm213 strain and the IHNV Sn1203 strain on day 45 post-vaccination. A significantly higher survival rate against IHNV was obtained in the rBlk94-VP2 group on day 45 post-vaccination (86%) compared with the PBS mock immunized group (2%). Additionally, IPNV loads decreased significantly in the rBlk94-VP2 immunized group in the liver (28.6-fold to 36.5-fold), anterior kidney (21.7-fold to 44.2-fold), and spleen (14.9-fold to 22.7-fold), as compared with the PBS mock control group. The mRNA transcripts for several innate and adaptive immune-related proteins (IFN-γ, IFN-1, Mx-1, CD4, CD8, IgM, and IgT) were also significantly upregulated after rBlk94-VP2 vaccination, and neutralizing antibodies against both IHNV and IPNV were induced on day 45 post-vaccination. Collectively, our results suggest that this recombinant virus could be developed as a vaccine vector to protect rainbow trout against two or more diseases, and our approach lays the foundations for developing live vaccines for rainbow trout.

1. Introduction

Rainbow trout, the most widely farmed cold-water fish in China, are important to the Chinese national economy. However, outbreaks of acute viral diseases have severely hampered breeding in rainbow trout. Infectious pancreatic necrosis virus (IPNV) and infectious hematopoietic necrosis virus (IHNV) are two common viral pathogens responsible for severe economic losses to the rainbow trout aquaculture industry (Xu et al., 2018, 2017b; Zhu et al., 2017).

IHNV, a *Novirhabdovirus* genus member of *Rhabdoviridae* family, contains a non-segmented single-stranded, negative-sense RNA molecule. The viral genome is approximately 11 kb long, and contains five structural proteins in the following order: a nucleocapsid protein (N), a polymerase-associated phosphoprotein (P), a matrix protein (M), a glycoprotein (G), a large RNA-dependent RNA polymerase (L), and a non-virion protein (NV) located between the G and L genes (Kurath et al., 2003). The G gene is widely used in evolution and phylogenetic studies on IHNV (Ahmadivand et al., 2017). Worldwide IHNV strains fall into five major genogroups (Jia et al., 2014). The genogroups U, M, and L predominantly occur in North America (Kurath et al., 2003; Purcell et al., 2011), and genogroup E is prevalent in European regions (Cieslak et al., 2017; Enzmann et al., 2005). Genogroup J, which was

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first reported in Japan, is believed to have evolved from the U genogroup (Kim et al., 2007; Nishizawa et al., 2006). Previous work from both our group and others revealed that IHNV isolates from seven Chinese provinces are all J genogroup members (Jia et al., 2014; Xu et al., 2018). Among the five genogroups, the M and J genogroup viruses are highly virulent in rainbow trout, while the U genogroup has significantly lower virulence relative to M, E and J IHNV genogroups in rainbow trout (Garver et al., 2006).

In contrast to IHNV, IPNV is an *Aquabirnavirus* genus member of the *Birnaviridae* family. IPNV contains a double-stranded segmented RNA molecule, containing segments A and B (Ahmadivand et al., 2018; Dobos and Roberts, 1983). The A segment is approximately 3 kb in size and contains two partially overlapping open reading frames (ORFs). The large ORF encodes the pVP2-VP4-VP3 polyprotein, which is cleaved by a non-structural protease (VP4) to generate two structural proteins, VP2 and VP3 (Xu et al., 2017b; Ji et al., 2017). The small ORF, which overlaps with the large ORF, generates VP5, a non-structural polypeptide. The VP2 protein contains conformational epitopes involved in viral antigenicity and virulence, which play a key role in inducing the host to produce immune-neutralizing antibodies (Heppell et al., 1995; Munang'Andu et al., 2013). VP3 is an internal structural protein in which some neutralizing epitopes have been identified (Ye et al., 2014). Based on these features, VP2 and VP3 are important target proteins for the development of vaccines against IPNV (Dadar et al., 2015; Munang'Andu et al., 2013). Segment B is approximately 2.8 kb long and encodes the VP1 RNA-dependent RNA polymerase protein (Ji et al., 2017).

IPNV and IHNV infections are widespread in Chinese rainbow trout hatcheries, and rainbow trout that survive infections with these viruses can carry these viruses for a long period of time after infection and may transmit them to other susceptible rainbow trout (Julin et al., 2015; Wargo et al., 2017); hence, preventing infections with these viruses is a worthwhile endeavor. Vaccines are considered to be an effective method for controlling such diseases, and some types of vaccines have been reported to protect rainbow trout against IHNV or IPNV, such as DNA vaccines (LaPatra et al., 2001; Xu et al., 2017b), attenuated vaccines (Fryer et al., 1976; Ristow et al., 2000; Rivas-Aravena et al., 2012), and inactivated viral vaccines (Anderson et al., 2008; Dixon and Hill, 2010). Among these vaccines, only one DNA vaccine against IHNV induces full disease protection and has been commercialized, but the vaccines against IPNV do not usually provide complete protection (Dhar et al., 2014; Ballesteros et al., 2014; Dadar et al., 2015). In a previous study, we designed a bivalent DNA vaccine against both IHNV and IPNV, which induced significant immune responses in rainbow trout (Xu et al., 2017b). Although this DNA vaccine induced effective protection, the feasibility of its application in China remains low because of the risk of genetic recombination within host cells. Therefore, it is necessary to develop more practical vaccines against both IHNV and IPNV.

The reverse genetics system is commonly tool used to investigate the gene functions and virulence mechanisms of negative-sense RNA viruses, and is also a powerful tool for producing recombinant viruses that can serve as attenuated vaccines (Guo et al., 2018; Romero et al., 2008; Rouxel et al., 2016; Thoulouze et al., 2004). In a previous study by Guo et al. (2018), two recombinant attenuated viruses were constructed against IHNV and IPNV, but the relative survival rate of the vaccinated host against IHNV challenge was only 60%, which is unlikely to meet the needs of rainbow trout aquaculture. Here, we constructed a new recombinant virus using U genogroup IHNV Blk94 as the vector, and inserted the VP2 gene from IPNV into the Blk94 genome between the P and M genes. We then assessed whether our recombinant virus could induce protective immune responses against both IPNV and IHNV infectious agents in rainbow trout to evaluate whether it might be a reliable candidate for the control of IPNV and IHNV infections in Chinese rainbow trout aquaculture.

2. Materials and methods

2.1. Ethics statement

The animal experiments were performed in accordance with the protocols approved by the Animal Welfare Committee of China Agricultural University (permit number: XK662), the guidelines and regulations of which were strictly followed throughout the study.

2.2. Fish, virus strains, and cell lines

Specific pathogen-free rainbow trout (mean weight: 5 ± 1 g) were purchased from a local farm with no record of IPNV and IHNV outbreaks in the past 3 years. Rainbow trout were collected from the farm and their tissue homogenates (liver, kidney and spleen) were inoculated into an IHNV and IPNV sensitive cell line to confirm that the experimental fish were pathogen-free by assessment of the cytopathic effect and PCR screening. The pathogen-free fish from the farm were kept at a constant temperature of 15 °C in separate 125 L aquaria. The IHNV-Blk94 strain (GenBank No: DQ164100), which was originally described by Dr. Gael Kurath (Western Fisheries Research Center, U.S. Geological Survey, Seattle, USA), was kindly provided by Dr. Hong Liu of the Shenzhen Entry-exit Inspection and Quarantine Bureau, China. The IHNV Sn1203 strain (IHNV-Sn1203; GenBank No: DQ164100) and IPNV ChRtm213 strain (IPNV-ChRtm213; GenBank No: KX234591) were isolated from rainbow trout hatcheries in China. Propagation and identification of the rescued recombinant viruses were assessed using the epithelioma *papulosum cyprini* (EPC) cell line (CRL-2872, ATCC).

2.3. Plasmid construction and recovery of recombinant IHNV

Total genomic RNA was extracted from Blk94 infected cells using TRIzol Reagent (15596026, Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The recombinant plasmid containing the full-length cDNA sequence from the Blk94 strain was generated using our previously reported method (Zhao et al., 2019a). Briefly, the entire genomic sequence of Blk94 was divided into five fragments, all of which were cloned into a modified pBluescript-based vector using the SuperScript III One-Step RT-PCR Platinum Taq HiFi Kit (12574030, Invitrogen). The resulting plasmid was named pIHNV-Blk94. The VP2 gene from IPNV-ChRtm213 was polymerase chain reaction (PCR)-amplified from the total genomic RNA from IPNV-ChRtm213 using the primers listed in Table 1. After identification on 1% agarose gels, the VP2 gene was purified and inserted into pIHNV-Blk94 between the P and M genes using the In-Fusion HD Cloning Plus Kit (638909, Takara, Japan). The final plasmid was sequenced and named pBlk94-VP2. The recombinant plasmid construction strategy is illustrated in Fig. 1, and the primers used for plasmid construction are shown in Table 1.

The recovery of recombinant IHNV was carried out using our previously reported method (Zhao et al., 2019a). Briefly, T7 RNA polymerase-expressing BHK-21 cells were transfected with a mixture of pBlk94-VP2 (0.5 μg), pIH-N (0.25 μg), pIH-P (0.25 μg), pIH-NV (0.05 μg), and pIH-L (0.1 μg) using Lipofectamine 2000 Reagent (1857332, Invitrogen). After incubation at 15 °C for 5 days, the cells were lysed using two freeze-thaw cycles, and the resulting virus was passaged in EPC cells. After three passages in EPC cells, the lysed cell supernatant collected by centrifugation was used as the rBlk94-VP2 stock.

2.4. Identification of rBlk94-VP2

2.4.1. Sequence identity of rBlk94-VP2

The viral RNAs from wildtype (wt) Blk94 or rBlk94-VP2 were separately extracted from the virus-infected cells using TRIzol Reagent. Reverse-transcriptase (RT)-PCR with primers FraAF and FraAR was employed to detect the VP2 gene. Fragment A (Fig. 1), which contains

Table 1
Primer sequences for pBlk94-VP2 plasmid construction and identification.

Primer	Primer name	Primer sequence (5'-3')
1	H1 F	CGACTCACTATAGGGGTATAAAAAAAGTAACTTGACTA
2	H1 R	TTCTTCACCTCTTGGGATCCTGCGTTGTCT
3	H2 F	AGGATCCCAAGAGGTGAAGAACATGGCCACT
4	H2 R	AGCCTTTGTGCATAGCGTAGACGTCATTTATT
5	H3 F	AAATGACGTCTACGCTATGCACAAAGGCTCCAT
6	H3 R	TGAGCGCTGTTTTTTGCATGACGCGTTCTA
7	H4 F	AGAACGCGTCATGCAAAAAACAGCGCTCACCCA
8	H4 R	TTGTGATTCCATGGGCATTGAGTAGAATTT
9	H5 F	AATGCCCATGGAATCACAACGGCCCCTTCA
10	H5 R	GGGACCATGCCGGCCGTATAAAAAAAGTAACAGAAGGGTT
11	IHNV-N F	AAACACGATAATACCATGACAAGCGCACTCAGAGAGACGT
12	IHNV-N R	TCGGATCTTAGGTCATCAGCGGAATGAATCGGAGTCTCCTGGCT
13	IHNV-P F	AAACACGATAATACCATGTCAGATGGAGAAGGAGAACA
14	IHNV-P R	TCGGATCTTAGGTCACTATTGACCTTGCTTCATGCGCTTCT
15	IHNV-NV F	AAACACGATAATACCATGGACCACTGTGACACAAACACGA
16	IHNV-NV R	TCGGATCTTAGGTCACTATCTGGGATAAGCAAGAAAGTCT
17	IHNV-L F	AAACACGATAATACCATGGACTTCTTCGATCTTGACATAGA
18	IHNV-L R	TCGGATCTTAGGTCACTATTGTTCGCCTAGTGGAAAGAA
19	FraAF	AAGCGGGCGGTGAGTCACGTCGGAGGAGA
20	FraAR	TCTCCTCCTCAACGTACTTTAGGATGAGTT
21	VP2F	AGTTCAAACGAGAGCATGAACACATCCAAGGCAACCGCAACT
22	VP2R	TCTTGAAAATAGACATGCTCTCGTTTGAACTGACTCTTGGA

N.b. The complete genome sequence of Blk94 was PCR-amplified using primers 1–10. The N, P, L, and NV gene-containing helper plasmids were constructed using primers 11–18. Primers 19 and 20 were used to PCR-amplify the sequences containing an engineered silent genetic tag. Primers 21 and 22 were used to PCR-amplify and identify the VP2 gene.

one silent mutation was PCR-amplified using primers FraAF and FraAR, and the resulting PCR products were purified and digested with *Nhe* I to differentiate the recombinant viruses from wtBlk94. Insertion of the VP2 gene into the rBlk94-VP2 genomic sequence was confirmed to have occurred in the viral RNA using the SuperScript III One-Step RT-PCR Platinum Taq HiFi Kit. All PCR products were analyzed using 1% agarose gels.

2.4.2. Indirect immunofluorescence assays and western blotting

EPC cells were infected with rBlk94, rBlk94-VP2, or phosphate-buffered saline (PBS) (mock infection). The virus-infected cells were cultured at 15 °C for 48 h. For the indirect immunofluorescence assays, the cells were fixed with 4% (w/v) paraformaldehyde. After fixation, the cells were permeabilized using 0.5% (v/v) Triton X-100, and then incubated with rabbit anti-IHNV-G antibody followed by Cy3-labeled goat anti-rabbit IgG secondary antibody (ab97075, Abcam, Cambridge, UK) to detect IHNV. The cells were also incubated with mouse anti-VP2 antibody followed by FITC-labeled goat anti-mouse IgG secondary antibody to detect the VP2 protein (ab6785, Abcam). The resulting images were observed on the DeltaVision workstation (GE Healthcare, USA). For western blotting, the cells were lysed with RIPA (P0013B, Beyotime, China) containing 1 mM PMSF (ST505, Beyotime). The lysates were separated on gradient polyacrylamide gels (4%–20%), and transferred onto nitrocellulose membranes (BS-NC-45, Biosharp, China). The membranes were incubated with either rabbit anti-IHNV-G antibody, mouse anti-VP2 antibody, or rabbit anti-β tubulin (ab 179513, Abcam) as the primary antibody, and then incubated with HRP-tagged secondary antibodies. The resultant images were observed with enhanced chemiluminescence (ECL) solution (34077, Thermo Scientific, Shanghai, China) using the ChemiScope 6000 Touch Imaging System (Clinx, Shanghai, China). The rabbit anti-IHNV-G antibody and mouse anti-VP2 antibody used in this study were obtained from previous research in our laboratory (Xu et al., 2017b).

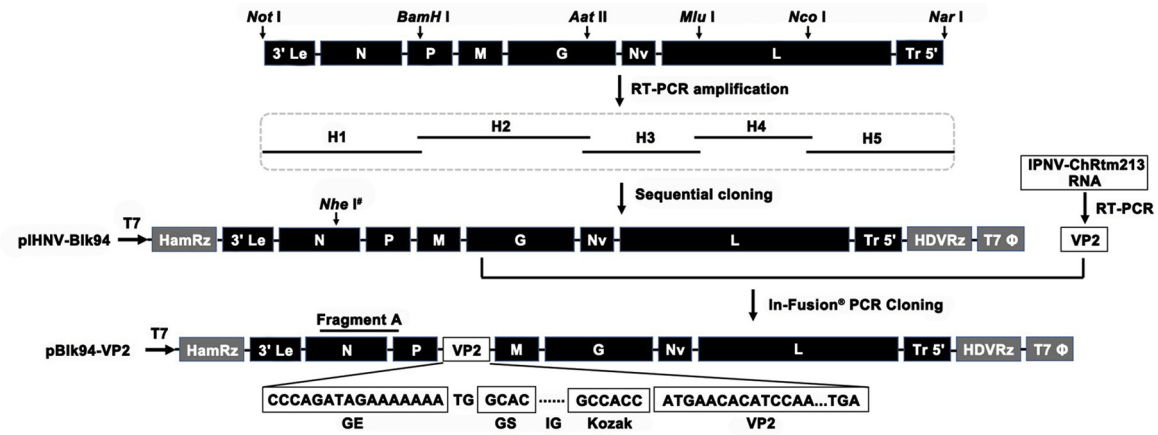


Fig. 1. Construction strategy for the complete Blk94-VP2 genome. The Blk94 viral genome sequence was divided into five fragments, all of which were cloned into a modified pBluescript-based vector. The vector contained the hammerhead ribozyme sequence (HamRz) and the hepatitis delta virus ribozyme sequence (HDVRz). By using site-directed mutagenesis, one additional unique restriction site (*Nhe* I) was added to the viral genome, which served as a molecular tag. The VP2 gene from IPNV was inserted between the P and M gene junction along with gene star (GS), intergenic (IG), and gene end (GE) sequences, which are necessary for the normal transcription and translation of the VP2 gene.

2.4.3. Virus growth kinetics

Supernatants from wtBlk94-, rBlk94-, and rBlk94-VP2-infected cells were collected at 12-h intervals and serially diluted ten-fold for viral titer determination by the $TCID_{50}$ method. EPC cell monolayers were first treated with different dilutions of the virus for 1 h, and then washed twice with PBS. The virus-infected cells were observed daily for 10 days to confirm the viral-induced cytopathic effect.

2.5. Pathogenicity assay

To determine the pathogenicity of rBlk94-VP2, rainbow trout were divided into five groups and maintained at 15 °C. Each group was split across three tanks (50 trout/tank). After keeping them in the tanks for 2 weeks, each rainbow trout was injected intraperitoneally with 50 μl of either wtBlk94, rBlk94, rBlk94-VP2, or IHNV-Sn1203, each at a dose of 2.0×10^2 pfu/mL, or with PBS (the mock infection control group). Mortality across all groups was monitored for 25 days.

2.6. Protective efficacy of rBlk94-VP2

Our previous study showed that measuring the cumulative percentage mortality or viral load after virus challenge were effective methods for assessing vaccine protective efficacy (Xu et al., 2017b). Because the IHNV-Sn1023 strain used in our study causes significant mortality and measuring the cumulative percentage mortality after virus challenge is the most direct, effective and widely used method, we only assessed the vaccine protective efficacy of the vaccine against IHNV using cumulative percentage mortality as the measure. However, the IPNV challenge infections in our study caused no mortality, so the protective efficacy of the vaccine against IPNV was assessed by measuring the IPNV viral load.

2.6.1. Challenge infections with IHNV-Sn1203 and cumulative percentage mortality

At day 45 post-immunization with 50 μl of rBlk94, rBlk94-VP2 (all viruses at a dose of 2.0×10^2 pfu/mL) or PBS (mock immunization), duplicate groups containing 50 rainbow trout each were injected intraperitoneally with IHNV-Sn1203 at a dose of 50 μl (2.0×10^2 pfu/mL). The mock infection group was injected with PBS (the same volume of fluid as for the other experimental groups). Mortality across all the groups was monitored for 25 days.

2.6.2. Challenge with IPNV- ChRtm213 and IPNV load measurements

On day 45 post-immunization with rBlk94, rBlk94-VP2 or PBS (mock immunization), the rainbow trout were challenged with the IPNV-ChRtm213 strain. Duplicate groups containing 50 rainbow trout each were injected intraperitoneally with IPNV-ChRtm213 at a dose of 1.0×10^6 pfu. The mock infection group was injected with the same volume of PBS. On day 15 post-challenge with IPNV-ChRtm213, the livers, anterior kidneys, and spleens from the challenged fish (n = 5) were collected and used to evaluate the efficacy of viral clearance and viral load as previously reported (Cuesta et al., 2010; Xu et al., 2017b). The RNA from each sample was extracted using TRIzol Reagent. The relative gene expression levels of IPNV VP1 and VP3 were analyzed with quantitative real-time polymerase chain reaction (RT-qPCR) using a RT-qPCR kit (RR096A, Takara, Dalian, China) and the primers listed in Table 2. The β-actin gene was used as an internal reference. The relative expression levels of all the analyzed genes were calculated using the $2^{-\Delta\Delta CT}$ method described previously (Zhao et al., 2017).

2.7. Immunity-related gene expression after rBlk94-VP2 immunization

To determine the immune response induced by rBlk94-VP2 vaccination, the mRNA expression levels of several immune genes in the liver, anterior kidney, and spleen were determined using the primers listed in Table 2. The mRNA levels of IFN-γ, IFN-1, and Mx-1, which are indicative of an innate immune response, were measured on days 1, 4, 7, and 15 post-immunization. The mRNA levels for IgM, IgT, CD4, and CD8, which are indicative of an adaptive immune response, were measured on days 15 and 21 post-immunization. The β-actin gene was used as an internal reference.

Table 2
Primer sequences for RT-qPCR.

Gene name	Primer name	Primer sequence (5'-3')
VP1	VP1 RT/F	ATCCTGCCCGCTAATGAATC
	VP1 RT/R	CGGCCTGTGCGGTGGTAGAT
VP3	VP3 RT/F	GCCGTTCGCATCTCACTGGA
	VP3 RT/R	GGTCGGCTTTGTTATGGTCTGT
IFN-1	IFN-1 RT/F	AGAATGCCCCAGTCCTTTTCC
	IFN-1 RT/R	GACTTTGTCCTCAAACTCAGCATCA
IFN-γ	IFN-γ RT/F	GTTGAGGGCCATGGATGTG
	IFN-γ RT/R	TCCAGCCCATCAAGCAGAA
Mx-1	Mx-1 RT/F	AGCGTCTGGCTGATCAGATT
	Mx-1 RT/R	AGCTGCTCGATGTTGTCCTT
IgM	IgM RT/F	CAAACCGGTGGAAGCTACAT
	IgM RT/R	AGACGGCTGCTGCAGATATT
IgT	IgT RT/F	AACATCACCTGGCACATCAA
	IgT RT/R	TTCAGGTTGCCCTTTGATTC
CD4	CD4 RT/F	CTGACCTCTGACCTGAAAGTG
	CD4 RT/R	TCCACAATTCACACCTCCAC
CD8	CD8 RT/F	GACTGCTGGCTGTGGCTTCC
	CD8 RT/R	CCCCGGAGCTGCCATTCT
β-Actin	β-Actin RT/F	GCCGGCCGCGACCTCACAGACTAC
	β-Actin RT/R	CGGCCGTGGTGGTGAAGCTGTAAC

2.8. Titration of neutralizing antibody

Blood samples from the vaccinated fish (n = 10) were collected from the caudal veins on day 45 post-vaccination, and the sera were isolated as previously described (Zhao et al., 2017). The neutralizing antibody titer of each sample, as determined via a neutralization assay, was defined as the reciprocal of the serum dilution that reduced the viral infectivity ($TCID_{50}$) by approximately 50% relative to that of the virus control. Serum samples from the mock immunized (with PBS) rainbow trout were used as negative control samples.

2.9. Statistical analyses

For multiple comparisons, ANOVAs were performed using GraphPad Prism (version 6) to analyze expression differences in the viral and immune-related genes between the rBlk94-VP2 vaccination and PBS mock vaccination groups. Differences between the two groups were determined using a Student's *t*-test. Kaplan-Meier survival curves were used to show the mortality profiles of wtBlk94, rBlk94, rBlk94-VP2, and IHNV-Sn1203 in the challenge infections. Values of $p < 0.05$ were considered indicative of statistical significance.

3. Results

3.1. Plasmid construction and recovery of rBlk94-VP2

The complete genomic sequence of Blk94 was PCR-amplified, cloned into a modified pBluescript-based vector, and the resulting recombinant plasmid was named pIHNV-Blk94. The recombinant plasmids pIHNV-Blk94, pIH-N, pIH-P, pIH-L, and pIH-NV were used to rescue the recombinant rBlk94 virus as described in the Methods section. The pIHNV-Blk94 plasmid was used as a backbone vector to construct the pBlk94-VP2 plasmid, which contains the VP2 gene inserted between the P and M genes. To ensure the normal transcription and translation of the VP2 gene, a gene junction sequence containing gene star, intergenic, and gene end components was added to the VP2 gene (Fig. 1). The recombinant pBlk94-VP2 plasmid was used to rescue

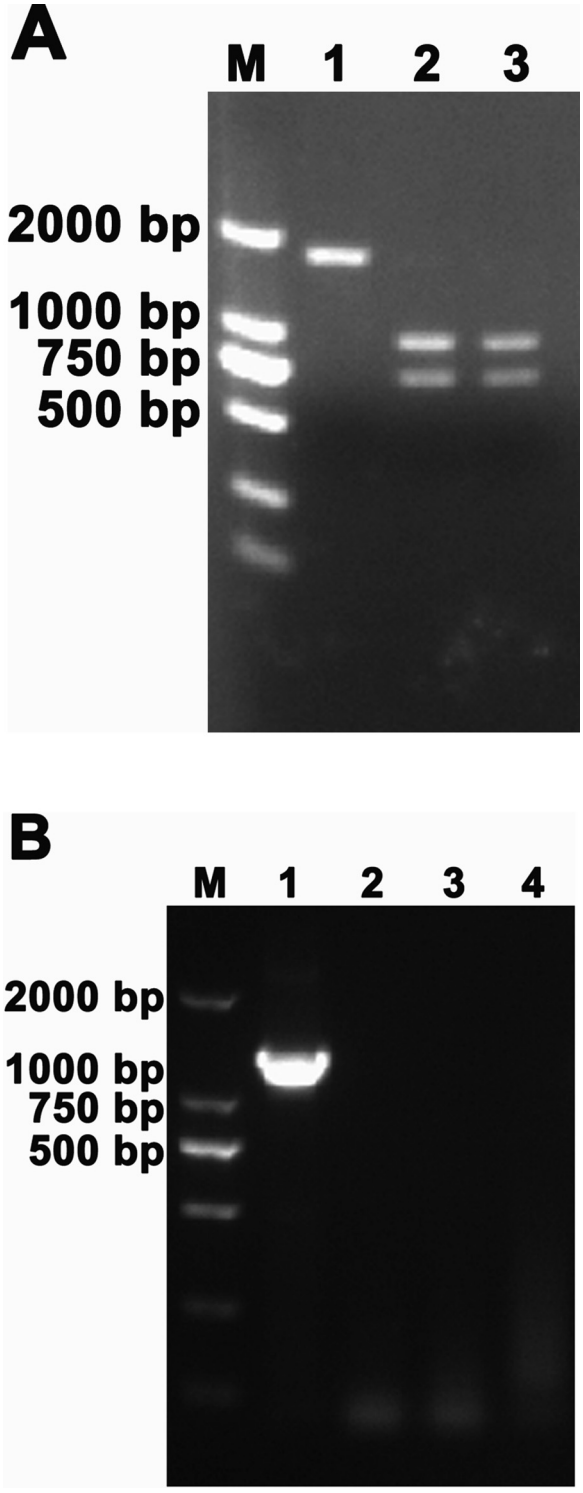


Fig. 2. Identification of rBlk94-VP2 using a genetic tag and the VP2 gene. Viral RNAs from wtBlk94, rBlk94, and rBlk94-VP2 were separately extracted from infected EPC cells 48 h after virus infection. (A) Genetic tag analysis of rBlk94-VP2. Lanes 1 to 3 show that fragment A was amplified from wtBlk94, rBlk94, or rBlk94-VP2, respectively, and then digested with *Nhe* I. (B) VP2 gene analysis of rBlk94-VP2. Lanes 1 to 4 show the PCR product analysis of rBlk94-VP2, wtBlk94, rBlk94, and the negative control, respectively, using the VP2 gene as the target gene.

the recombinant rBlk94-VP2 virus.

3.2. Identification of rBlk94-VP2

3.2.1. Identification of the recombinant virus using silent genetic tags and the VP2 gene

To differentiate the recombinant viruses (rBlk94 and rBlk94-VP2) from the wtBlk94 virus, one silent genetic tag (*Nhe* I digestion site) was added to the rBlk94 and rBlk94-VP2 viral genomes using site-directed mutagenesis. Fragment A was amplified from the viral genomic RNA by RT-PCR (as shown in Fig. 1). The resulting PCR products were purified and digested with *Nhe* I. The results showed that the PCR products from rBlk94 and rBlk94-VP2 were digested by *Nhe* I, but the PCR product from wtBlk94 was not digested by this restriction endonuclease (Fig. 2A). Additionally, the VP2 gene was successfully PCR-amplified from rBlk94-VP2 viral genomic RNA (Fig. 2B). The full genome sequence identities and stable insertion of the VP2 gene from rBlk94-VP2 were both determined after six passages, and no genetic mutations were founded when compared with wtBlk94, except for the artificially introduced silent genetic tag and the inserted VP2 gene (data not shown).

3.2.2. Identification of rBlk94-VP2 by indirect immunofluorescence assay and western blotting

Indirect immunofluorescence assay and western blotting were both employed to confirm the successful expression of the VP2 gene by rBlk94-VP2. Mouse anti-VP2 primary antibody and FITC-labeled goat anti-mouse IgG secondary antibody were used to detect VP2 protein expression in the rBlk94-VP2 virus. The presence of the rBlk94 virus was detected using rabbit anti-IHNV-G antibody and Cy3-labeled goat anti-rabbit IgG secondary antibody. At 48 h post-infection with rBlk94 or rBlk94-VP2, the EPC cells were observed with an immunofluorescence microscope (a DeltaVision workstation). The results showed specific red and green fluorescence in the rBlk94-VP2-infected cells, but only red fluorescence in the rBlk94-infected cells, and no specific fluorescence was observed in the PBS-treated cells (Fig. 3A). On western blotting, a specific protein band expressed by rBlk94-VP2-infected cells was visible after incubation with the mouse anti-VP2 antibody, unlike the results from the rBlk94-infected and PBS mock infected cells where no bands were produced (Fig. 3B). These data suggest that the VP2 gene was successfully expressed in the rBlk94-VP2 virus.

3.2.3. Virus growth kinetics

The supernatants from cells infected with wtBlk94, rBlk94, or rBlk94-VP2 were collected at 12-h intervals and used for an in vitro virus growth kinetics study. As expected, both the rBlk94 and rBlk94-VP2 viruses showed similar growth curves compared with the wtBlk94 virus, and all three of these viruses reached a stable phase by 72 h post-infection (Fig. 4). This result indicates that there were no significant differences in viral replication among the three viruses.

3.3. Pathogenicity analysis of rBlk94-VP2

A virus challenge experiment was performed to determine the pathogenicity of wtBlk94, rBlk94 and rBlk94-VP2 in rainbow trout, and fish mortality was monitored daily for 25 days. Because we reported in a previous study that IHNV-Sn1203 is a virulent strain (Zhao et al., 2019a), we used it as a positive control in the present study. Rainbow trout began to die on the fourth day after challenge with IHNV-Sn1203, and the peak death rate occurred between days 6 and 11 post-challenge. The cumulative mortality was 96% in the IHNV-Sn1203-challenged group after 25 days. In contrast, the cumulative mortality was only 4%–8% in the wtBlk94, rBlk94, and rBlk94-VP2 challenged groups (Fig. 5). This suggests that rBlk94-VP2 is a recombinant virus that has a low virulence profile not dissimilar to the wtBlk94, making it worth investigating for its potential as a candidate vaccine against IHNV infection.

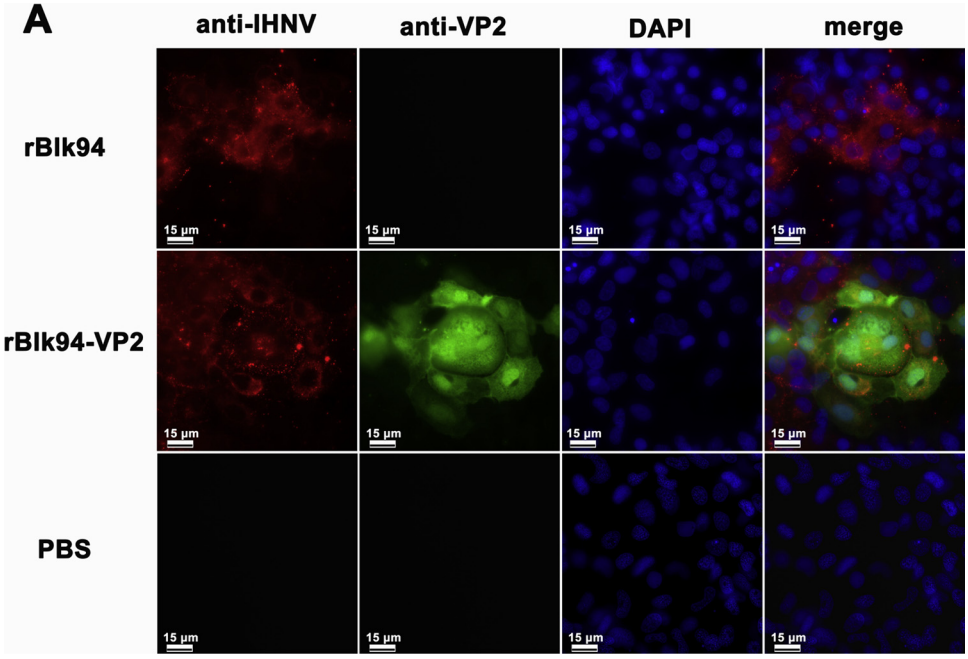
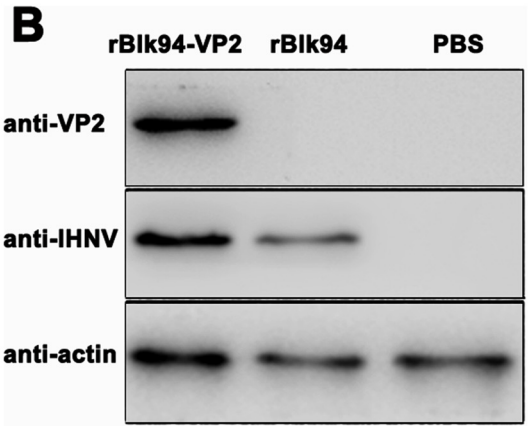


Fig. 3. Analysis of rBlk94-VP2 by indirect immunofluorescence assay and western blotting. EPC cells were infected with rBlk94, rBlk94-VP2 or PBS (mock infection) for 48 h. (A) The cells were incubated with mouse anti-VP2 antibody followed by FITC-labeled goat anti-mouse IgG secondary antibody to detect the VP2 protein (green), as well as with rabbit anti-IHNV-G antibody followed by Cy3-labeled goat anti-rabbit IgG secondary antibody to detect IHNV (red). Cell nuclei were stained with DAPI (blue). (B) Cells were lysed with RIPA containing 1 mM PMSF and their protein contents were separated on polyacrylamide gels, and then transferred onto nitrocellulose membranes. The membranes were incubated with the primary antibody, either rabbit anti-IHNV-G antibody, mouse anti-VP2 antibody, or rabbit anti-β tubulin (used as the internal control), and then incubated with an HRP-tagged antibody. The images were observed using ECL solution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).



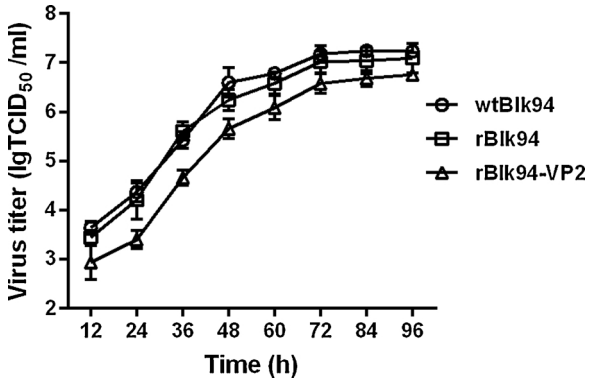


Fig. 4. Growth kinetics of rBlk94-VP2. The supernatants from cells infected with different viruses (rBlk94, rBlk94-VP2, or wtBlk94) were harvested at 12-h intervals in three independent experiments. The viral titers at different time points were determined by $TCID_{50}$ measurements. Differences in the viral titers were analyzed using ANOVAs with GraphPad Prism (version 6) for multiple comparisons, and Student's *t*-tests were used to assess any differences between the two groups at a single time point. Values where of $p < 0.05$ were considered indicative of statistical significance.

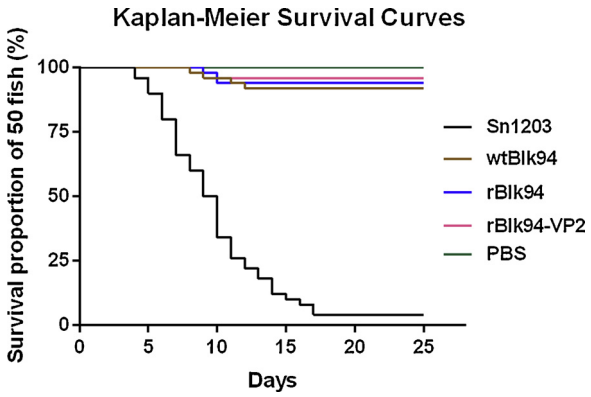


Fig. 5. Pathogenicity analysis of rBlk94-VP2. Rainbow trout were injected intraperitoneally with 50 μL of wtBlk94, rBlk94, rBlk94-VP2, or IHNV-Sn1203 (all viruses were at doses of 2.0×10^2 pfu/mL), and PBS was used as the mock infection control. Mortality across all of the infected groups was monitored daily for 25 days.

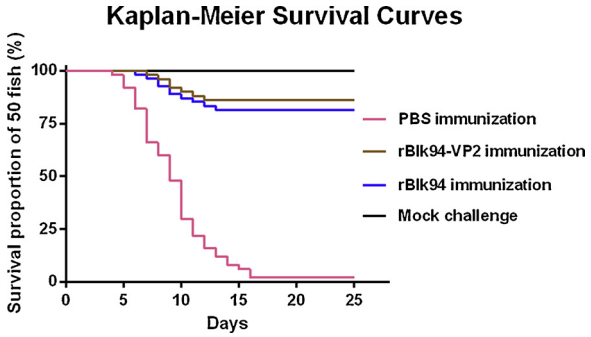


Fig. 6. Survival rate of rainbow trout immunized with rBlk94-VP2 and challenged with the Sn1023 strain. Rainbow trout were immunized with 50 µl of rBlk94, rBlk94-VP2 or PBS (mock immunization), and then challenged with the IHNV-Sn1203 strain (all viruses were used at a dose of 2.0×10^2 pfu/mL) by intraperitoneal injection at 45 days post-immunization. The mock challenge group was injected with the same volume of PBS instead of IHNV-Sn1203. Mortality across all of the challenged groups was monitored daily for 25 days.

3.4. Protective efficacy of rBlk94-VP2

3.4.1. Protection by rBlk94-VP2 against IHNV-Sn1203 in rainbow trout

On day 45 post-immunization with rBlk94, rBlk94-VP2 or PBS (mock immunization), 50 fish in each group were challenged with IHNV-Sn1203. Rainbow trout that were vaccinated with rBlk94 or rBlk94-VP2 had survival rate of 80% and 86%, respectively. The survival rates for trout vaccinated with rBlk94 or rBlk94-VP2 were both significantly higher than that for those vaccinated with PBS (survival rate: 2%), but there was no significant difference between the rBlk94 and rBlk94-VP2 group (Fig. 6). There was no mortality in the mock challenge group, and there were no significant differences between any of the replicates (data not shown).

3.4.2. Decreased IPNV load in rainbow trout

Three groups of rainbow trout (n = 50) were each challenged with the IPNV-ChRtm213 strain on day 45 post-immunization with rBlk94, rBlk94-VP2 or PBS (mock immunization). Because the IPNV strain used for the challenge infections in this study caused no mortality (previously reported by Xu et al., 2017b), the viral load was measured to assess each vaccine's protective efficacy. The VP3 and VP1 gene expression levels in the liver, anterior kidney, and spleen were measured to evaluate the viral load at 15 days post-challenge with IPNV. The IPNV loads in the rBlk94-VP2 immunization group was significantly lower than those in the rBlk94 group and PBS group. Additionally, the average fold changes of the VP3 and VP1 genes were respectively 36.5-fold and 28.6-fold (in the liver), 44.2-fold and 21.7-fold (in the anterior kidney), and 14.9-fold and 22.7-fold (in the spleen) lower in the rBlk94-VP2 group than those in the PBS group. The average fold changes of the VP3 and VP1 genes were respectively 37.9-fold and 25.4-fold (in the liver), 37.5-fold and 19.7-fold (in the anterior kidney), and 13.8-fold and 19.6-fold (in the spleen) lower in the rBlk94-VP2 group than those in the rBlk94 group. However, no significant VP3 and VP1 fold changes were found between the rBlk94 and PBS groups. Collectively, these results suggest that the IPNV load was significantly reduced in rainbow trout that were immunized with rBlk94-VP2 compared with those immunized with rBlk94 or were mock vaccinated with PBS (Fig. 7).

3.5. Immunity-related gene expression after rBlk94-VP2 immunization

To further investigate the immune response after rBlk94-VP2 immunization, the relative gene expression levels of IFN-γ, IFN-1, Mx-1, CD4, CD8, IgM, and IgT were evaluated in the livers, anterior kidneys, and spleens from the rainbow trout. The mRNA expression levels of the genes for innate immunity proteins (IFN-γ, IFN-1, and Mx-1) were measured on days 1, 4, 7, and 15 post-vaccination, and the results

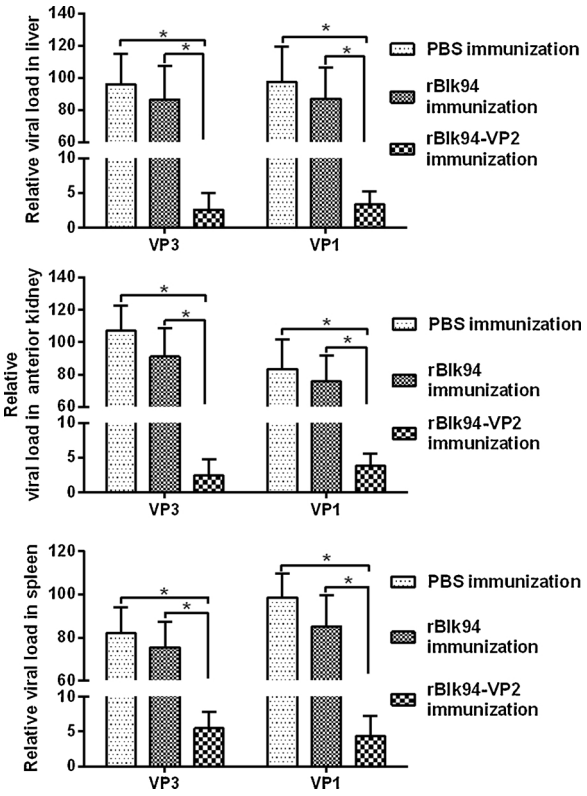


Fig. 7. Determination of the IPNV load by RT-qPCR. Rainbow trout were immunized with rBlk94, rBlk94-VP2, or PBS (mock immunization), and then challenged with the IPNV-ChRtm213 strain at a dose of 1.0×10^6 pfu by intraperitoneal injection at 45 days post-immunization. At 15 days post-challenge with IPNV-ChRtm213, the relative VP3 and VP1 gene expression levels in the livers, anterior kidneys, and spleens of the rainbow trout were analyzed to evaluate the efficacy of viral clearance and the viral load.

showed that all three of these genes were significantly upregulated in rBlk94 and rBlk94-VP2 vaccination groups when compared with the PBS group (Fig. 8). The highest fold changes for IFN-γ (14.9-fold in the liver, 28.4-fold in the anterior kidney, and 12.1-fold in the spleen), and IFN-1 (9.9-fold in the liver, 6.6-fold in the anterior kidney, and 5.8-fold in the spleen) were both found on day 1 post-vaccination with rBlk94-VP2, while the highest fold changes for Mx-1 in the rBlk94-VP2 group were found on day 7 post-vaccination in the liver (45.1-fold) and on day 4 post-vaccination in the anterior kidney and spleen (28.2-fold and 22.5-fold, respectively) compared with the PBS group. The mRNA levels of the adaptive immunity proteins (CD4, CD8, IgM, and IgT) were measured on days 15 and 21 post-vaccination (Fig. 9). The results revealed that all four of these genes in the rBlk94-VP2 vaccination group were upregulated on day 15 post-vaccination, and the highest fold changes for CD4 (1.9-fold and 16.3-fold), CD8 (1.3-fold and 28.7-fold), IgM (3.0-fold and 36.3-fold), and IgT (1.6-fold and 9.3-fold) occurred on day 21 post-vaccination in the anterior kidney and spleen, compared with the PBS group. However, in the liver, the highest fold changes for CD4 (2.8-fold), CD8 (8.3-fold), and IgM (4.2-fold) were obtained on day 15 post-vaccination, and that of IgT (2.6-fold) was obtained on day 21 post-vaccination compared with the PBS group. These results suggest that innate and adaptive immune responses were both activated by vaccination with rBlk94-VP2. However, no significant differences were found between the rBlk94 and rBlk94-VP2 vaccination groups in terms of the expression of these immune-related genes.

3.6. Neutralizing antibody analysis

Serum samples from ten rainbow trout in the rBlk94-VP2-

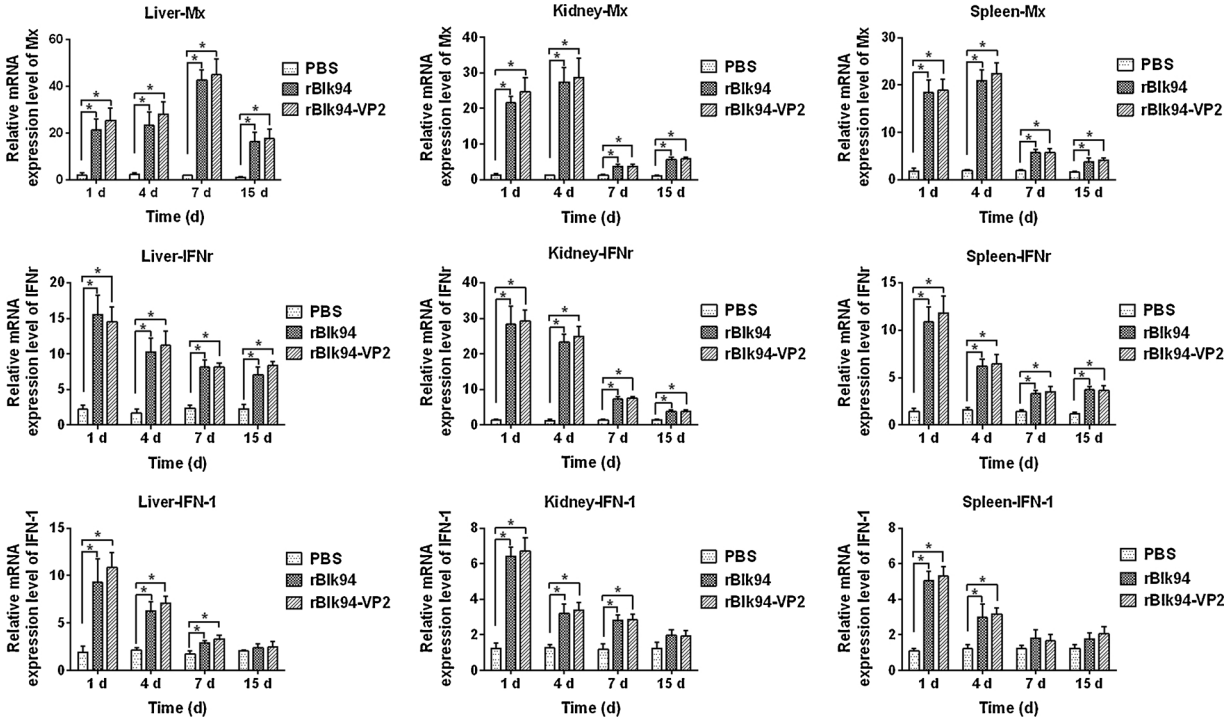


Fig. 8. Evaluation of the innate immunity related genes induced by rBlk94-VP2 immunization. Rainbow trout were immunized with rBlk94, rBlk94-VP2 or PBS (mock immunization). The mRNA expression levels of the innate immunity proteins IFN-γ, IFN-1, and Mx-1 were measured on days 1, 4, 7, and 15 post-vaccination. The mRNA expression levels of all the targeted genes were normalized to that of the β-actin gene. The standard deviations of the means (n = 5) are represented as error bars.

vaccinated group were tested for neutralizing antibodies against IPNV and IHNV. Most of the rBlk94-VP2-vaccinated rainbow trout had high neutralizing antibody titers (≥160) against both IHNV and IPNV, whereas no specific neutralizing antibodies were found in the PBS vaccinated group (Table 3).

4. Discussion

Rainbow trout is the most widely farmed cold-water fish species in China, and most Chinese aquaculture factories raise diploid and triploid rainbow trout. IHNV and IPNV have been the main pathogens responsible for severe disease in rainbow trout since the first outbreaks of these two diseases in northeast China in 1985 and 1986, respectively (Xu et al., 2017b; Zhu et al., 2017). Both of these viruses have evolved over many years since their first detection in China. IHNV strains from around the world are divided into five major genogroups: U, M, L, E, and J, and the Chinese IHNV isolates have all been found to belong to genogroup J (Xu et al., 2017a). Previous studies have found that J genogroup viruses inflict a mortality rate exceeding 90% in rainbow trout, whereas the U genogroup is of significantly lower virulence in these fish (Penaranda et al., 2009; Xu et al., 2017a). Although the most effective vaccines against IHNV and IPNV that have been reported to date are DNA vaccines, the safety of such DNA vaccines remains controversial because of their potential risks, such as chromosomal integration and immune tolerance against the expressed antigen (Dadar et al., 2015). Therefore, other vaccines to protect rainbow trout against IHNV and IPNV still need to be developed. Previous work showed that the reverse genetics system is a powerful tool for producing multivalent recombinant viruses that can serve as live vaccines against two or more pathogens (Emmenegger et al., 2018; Hu et al., 2018). Because most of the farmed cold-water fish in China are rainbow trout, and the U genogroup strain Blk94 has relatively low virulence in rainbow trout, we believed it could be used as a live vaccine vector by expressing an antigenic protein from IPNV to protect rainbow trout against both IPNV and IHNV.

IPNV is a member of the genus *Aquabirnavirus* in the family *Birnaviridae*, and aquatic birnaviruses are believed to be antigenically variable (Frost et al., 1995). Therefore, a vaccine against IPNV should be based on the neutralization epitopes that are conserved among the relevant viral strains. The major serotype-specific neutralization epitopes in aquatic birnaviruses are located in the VP2 protein, and this protein can induce neutralizing antibodies to protect the host against viral infection (Fridholm et al., 2007; Frost et al., 1995). Based on both of these published studies, we constructed a recombinant virus containing the VP2 gene in the viral genome. It is widely accepted that nonsegmented, single-stranded, negative-sense viruses synthesize and transcribe viral genes into mRNAs in a sequential and polar manner via a stop-and-restart mechanism at each gene junction (Zhao et al., 2015). Therefore, the promoter-proximal genes are expressed more efficiently than the promoter-distal ones. However, a foreign gene inserted in the non-coding region between the N and P genes was found to severely impair viral growth, indicating that the junction between the N and P genes may not be the best site for foreign gene expression (Zhao et al., 2019b, 2015). Instead, we chose the P and M junction as the insertion site for VP2 gene expression in the recombinant rBlk94-VP2 virus. Although Guo et al. (2018) constructed a recombinant attenuated IHNV that expresses the VP2 protein from IPNV, immunization with this recombinant virus still caused a cumulative mortality rate of 5% in rainbow trout, and the relative percentage survival rate was only 65% (Guo et al., 2018). The recombinant rBlk94-VP2 virus constructed in our study caused only 2%–4% mortality after vaccination, and the relative survival rate for rBlk94-VP2-vaccinated trout was 86%; hence, our new vaccine candidate is more effective than the one from the previous study. There are several possible explanations for the relatively higher protection induced by rBlk94-VP2. First, the previous study replaced the NV gene with the VP2 gene from IPNV. This exchange severely impaired viral growth such that the vaccine candidate may not have provided enough immunogenicity to induce a strong

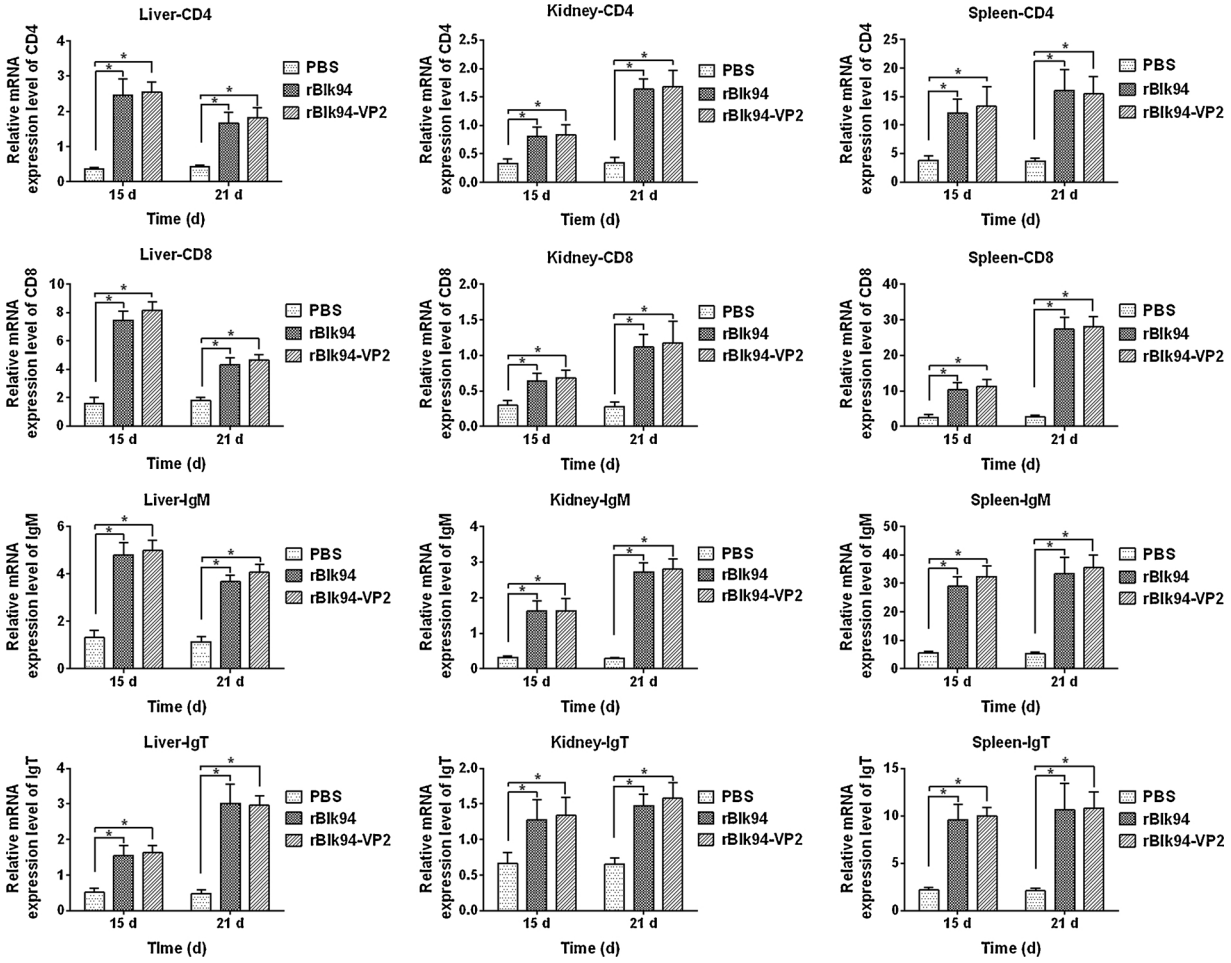


Fig. 9. Evaluation of the adaptive immunity related genes induced by rBlk94-VP2 immunization. Rainbow trout were immunized with rBlk94, rBlk94-VP2 or PBS (mock immunization). The mRNA expression levels of the adaptive immunity proteins CD4, CD8, IgM, and IgT were measured on days 15 and 21 post-vaccination. The mRNA expression levels of all the targeted genes were normalized to that of the β-actin gene. The standard deviations of the mean values (n = 5) are represented as error bars.

Table 3
IPNV and IHNV neutralizing antibody titers induced by rBlk94-VP2 infection.

Neutralizing antibody titers-IPNV				Neutralizing antibody titers-IHNV			
rBlk94-VP2 vaccinated		PBS vaccinated		rBlk94-VP2 vaccinated		PBS vaccinated	
Fish	Titer	Fish	Titer	Fish	Titer	Fish	Titer
rB1	80	P1	10	rB1	160	P1	10
rB2	160	P2	10	rB2	160	P2	10
rB3	160	P3	10	rB3	80	P3	10
rB4	320	P4	10	rB4	320	P4	10
rB5	80	P5	5	rB5	160	P5	10
rB6	160	P6	10	rB6	320	P6	5
rB7	160	P7	5	rB7	160	P7	10
rB8	160	P8	5	rB8	80	P8	10
rB9	320	P9	10	rB9	160	P9	10
rB10	160	P10	10	rB10	320	P10	10

immune response in rainbow trout (Thoulouze et al., 2004). Second, the VP2 gene was inserted between the G and L gene in the previous work, and this insertion site, which is further away from the promoter-proximal genes compared with the P and M junction, may not express enough VP2 protein to stimulate the host to produce a robust immune response against IPNV infection.

Here, a recombinant IHNV vaccine with relatively low virulence was constructed using the U genogroup virus Blk94 as a backbone vector, and the VP2 gene was inserted into the viral genome at the location believed to be the best insertion site for successful foreign gene expression (Zhao et al., 2019b, 2015). The rBlk94-VP2 virus shared similar growth kinetics with wtBlk94, and the immunofluorescence assay results confirmed that the VP2 gene was successfully expressed by the rBlk94-VP2 virus. The protective efficacy of this lower virulence vaccine was also assessed by viral challenge. Rainbow trout that were immunized with the vaccine had a higher survival rate (86%) than those in the PBS mock vaccination group (2%) after challenge infection with the virulent IHNV-Sn1203 strain. Cumulative percentage mortality measurement is the most direct, effective and widely used method for assessing a vaccine's protective efficacy. However, the IPNV strain used for challenge caused no mortality in the present study, or in our previous study (Xu et al., 2017b), and other reported studies (de Las et al., 2009; Dhar et al., 2010, and Martinez-Alonso et al., 2012) all found that viral load measurement is also an effective way to assess a vaccine's protective efficacy. Therefore, the viral loads and neutralizing antibody levels were measured to assess the protective efficacy of the vaccine. The IPNV load was dramatically lower and a high level of neutralizing antibody against IPNV was generated in the rBlk94-VP2 vaccination group compared with the PBS mock vaccination group. The gene expression levels of IFN-γ, IFN-1, Mx-1, CD4, CD8, IgM, and IgT were also significantly upregulated in the rBlk94-VP2-vaccinated trout, suggesting that both innate and adaptive immune responses were activated by rBlk94-VP2 vaccination.

The most commonly researched vaccines for rainbow trout are DNA

vaccines, which reportedly afford protection rates of 83%–98% against the lethal effects of IHNV challenge (Zhao et al., 2017; Alonso and Leong, 2013). In contrast, inactivated viral vaccines are reported to induce protection rates of 50%–79% in vaccinated rainbow trout against IHNV challenge (Anderson et al., 2008). Both forms of these vaccines are also reported to induce a good level of protective immunity against IPNV infection (Ahmadivand et al., 2018; Cuesta et al., 2010; Rivas-Aravena et al., 2012). The live, recombinant, relatively low virulence vaccines constructed by Guo et al. (2018) and ourselves were found to protect 65%–86% of the vaccinated rainbow trout against IHNV challenge. Although their protective efficacies are somewhat lower than that of DNA vaccine, they could still be promising vaccine candidates, because the most effective immunization delivery method for DNA vaccination is injection, it is labor intensive and impractical for large-scale administration in the commercial freshwater aquaculture industry, whereas a live recombinant virus has potential to obtain a similar efficacy by water immersion. Although previous studies and our results show that a live recombinant virus with relatively low virulence would be a reliable candidate for a vaccine aimed at controlling IHNV infection, several ethical issues need consideration. First, although the genetic character of the recombinant virus vaccine strain is relatively stable, virus atavism may occur via repeated inoculation and passage. Second, although a recombinant vaccine may be hypovirulent in certain immunized animals, it could be dangerous to other animals that are highly susceptible to such a strain. Third, the immune efficacy may be negatively affected if the recipient fish are undergoing drugs treatment during immunization with the recombinant virus. Finally, the live virus vaccines are unstable and difficult to store and transport. Considering these concerns, more work will need to be done before a live recombinant virus will be ready to be developed as a vaccine for clinical use.

In conclusion, we constructed a recombinant IHNV, rBlk94-VP2 vaccine, based on the U genogroup virus Blk94, a virus with relatively low virulence in rainbow trout. The recombinant virus showed the same growth kinetics and pathogenicity as the wtBlk94 strain and induced significant protective efficacy against both IHNV and IPNV in rainbow trout. That the VP2 gene was successfully expressed by the rBlk94-VP2 virus suggests that this recombinant IHNV with relatively low virulence could be a useful vaccine vector for protecting rainbow trout against two or more diseases. Our approach also provides a firm foundation for the development of live vaccines for use in rainbow trout.

Declaration of Competing Interest

The authors declare that they have no conflict of interest related to this work.

Acknowledgements

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