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Protection of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus (IHNV) by immunization with G gene's cytoplasmic and transmembrane region-deleted single-cycle IHNV

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Single-cycle viruses generated by reverse genetic technology are replication-incompetent viruses due to the elimination of gene(s) essential for viral replication, which provides a way to overcome the safety problem in attenuated viruses. Infectious hematopoietic necrosis virus (IHNV) is a major pathogen causing severe damage in cultured salmonid species. In the present study, we generated a single-cycle IHNV lacking the transmembrane and cytoplasmic domain in the G gene (rIHNV-GΔTM) and evaluated the prophylactic potential of rIHNV-GΔTM in rainbow trout (*Oncorhynchus mykiss*). To produce rIHNV-GΔTM, IHNV G protein-expressing Epithelioma papulosum cyprini (EPC) cells were established. However, as the efficiency of rIHNV-GΔTM production in EPC cell clones was not high, fish were immunized with a low-tittered single-cycle virus (1.5×10^2 PFU/fish). Despite the low dose, the single-cycle IHNV induced significant protection in rainbow trout against IHNV infection, suggesting high immunogenicity of rIHNV-GΔTM. No significant difference in serum ELISA titers against IHNV between the rIHNV-GΔTM immunized group and the control group suggests that the immunized dose of rIHNV-GΔTM might be too low to induce significant humoral adaptive immune responses in rainbow trout. The involvement of adaptive cellular immunity or innate immunity in the present significantly higher protection by the immunization with rIHNV-GΔTM should be further investigated to know the protection mechanism.

Key words: Single-cycle virus, IHNV, rIHNV-GΔTM, Immunization, Rainbow trout.

Introduction

Various piscine rhabdoviruses have had a devastating effect on aquaculture farms, and many of them are included in the notifiable diseases in the World Organization for Animal Health (OIE). Infectious hematopoietic necrosis virus (IHNV) belonging to the genus *Novirhabdovirus* in the family *Rhabdoviridae* is a major pathogen causing severe damage in cultured salmonid species including Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) worldwide (Amend *et al*., 1969; Dixon *et al*., 2016; Kurath, 2012). Although IHNV G protein-based DNA vaccine was the first permitted DNA vaccine in animals and the high protective efficacy of the DNA vaccine against IHNV infection has been proved (Anderson *et al*., 1996; Lorenzen *et al*., 2002; Yong *et al*., 2019), the permission for commercialization of DNA vaccines in cultured fish is still very

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struggling in many countries due to the concerns about environmental risk and safety related to gene-modified organisms (GMOs) issue (Alonso and Leong, 2013).

The rescue of recombinant viruses using reverse genetic technology has become a way to produce artificially attenuated viral vaccines (Biacchesi, 2011; Stobart and Moore, 2014). As the exact engineering of viral target genes is possible, artificially attenuated viruses generated through reverse genetic technology are usually safer than naturally attenuated viruses produced through long-term successive in vitro passages. However, the commercial application of artificially attenuated viruses is limited due to not only the possible epizootics in immunologically compromised individuals but also being regarded as GMOs.

Recombinant IHNVs have also been produced using reverse genetics (Ammayappan *et al*., 2010; Biacchesi *et al*., 2000; Zhao *et al*., 2019), and have been applied for prophylactic vaccines (Guo *et al*., 2018; Romero *et al*., 2008; Rouxel *et al*., 2016). However, the maintenance of replication ability in those recombinant IHNVs can be a bottleneck to get permission as commercial vaccines. Single-cycle viruses that are lacking in a gene or genes essential for viral replication can be a way to overcome the safety problem in attenuated viruses. Although gene(s)-deleted single-cycle viruses are still treated as GMOs, no addition of heterologous nucleotides and no ability to produce replication-competent viruses can minimize risks and can make them easier for getting permission as commercial vaccines. Previously, we had rescued a full ORF of G gene-deleted or a G gene's transmembrane-cytoplasmic region-deleted single-cycle VHSV (rVHSV-ΔG and rVHSV-GΔTM, respectively), and had demonstrated the high potential of the replication-deficient single-cycle VHSVs as prophylactic vaccines (Kim and Kim, 2019; Kim *et al*., 2015). However, there has been no available information on the production of single-cycle IHNV and the protective ability of single-cycle IHNV-based vaccines. Therefore, the purpose of the present study was to know whether single-cycle IHNV can induce protection in rainbow trout like single-cycle VHSVs. For this, we newly generated a single-cycle IHNV lacking the transmembrane and cytoplasmic domain in the G gene (rIHNV-GΔTM) and evaluated the prophylactic potential of rIHNV-GΔTM in rainbow trout.

Materials and methods

Cells and virus

Epithelioma papulosum cyprini (EPC) cells were grown in Leibovitz L-15 medium (L-15, Sigma) supplemented with 1% penicillin-streptomycin (Welgene) and 10% fetal bovine serum (FBS, Welgene) at 28 ℃. IHNV RtWanju-15 (genotype JRt Nagano lineage; isolated from cultured rainbow trout in Korea) was propagated in EPC cells supplemented with antibiotics and 2% FBS at 15 ℃. When broad cytopathic effect (CPE) was observed, the culture supernatant was collected by centrifugation, then kept in a deep freezer at -80 ℃ until use.

Construction of plasmids

Total RNA was isolated from wild-type IHNV stock using the Hybrid-R kit (GeneAll, Korea), and was converted into cDNA using the reverse transcription kit (Elpisbio, Korea) according to the manufacturer's instructions. PCR primer sets (Table 1) were designed to produce six partially overlapping fragments covering IHNV full genome, and each PCR-amplified fragment was cloned into the pGEM T-easy vector (Promega) and sequenced. The backbone vector sequence containing CMV promoter, hammerhead ribozyme (HHR) and hepatitis delta virus ribozyme (HDV) was PCR amplified using a previously constructed vector, pSHRV-Gvhsv (Lee et al., 2021), as the template, and the HHR guide RNA sequence was modified by site-directed mutagenesis to fit IHNV (primers in Table 1). All PCR amplified fragments were treated with *Dpn*I (Elpisbio) and were

Table 1. Primers used in this study

Name	Sequence (5'-3')
For construction of rIHNV-wild	
IHNV_1_F	GTATAAGAAAAGTAACTTGACTAAGCTCAGAAGGAC
IHNV_3937_R	CATTCCTCTCTGCTCTCCGAGG
IHNV_3930_F	GAGCAGAGAGGAATGTCTTGAGG
IHNV_6018_R	CTCTTGAGAAGACACGTGGGTACG
IHNV_6004_F	GTGTCTTCTCAAGAGACACCATCCAG
IHNV_8042_R	GATGTTGGTGGCTCCTGCAAG
IHNV_7476_F	GACCTGGCGACTCATCAGAC
IHNV_9761_R	CTTCCAGGGGGTGTCTCCAATG
IHNV_9010_F	CCTACTTCACCACCTGGTGTG
IHNV_11133_R	TGTATAAAAAAAGTAACAGAAGGGTTCTCAAAAGGC
IHNV_OC_F1	GACGTCTACGCTATGGGGTCGGCATGGCATCTCC
IHNV_OC_R1	GTTACTTTTCTTATACCCTATAGTGAGTCGTATTAGCCGGC
IHNV_OC_F2	CATTCCTCTCTGCTCTCCGAGG
IHNV_OC_R2	CATTCCTCTCTGCTCCCTATAGTGAGTCGTATTAGCCGGC
IHNV_OC_F3	GTTACTTTTTTTATACAGGGTCGGCATGGCATCTCC
IHNV_OC_R3	GATGAGTCGCCAGGTCCCTATAGTGAGTCGTATTAGCCGGC
IHNV_OC_F4	CATTCCTCTCTGCTCCATAGCGTAGACGTCATTTATTCCGGG
IHNV_OC_R4	GTTACTTTTTTTATACAGGGTCGGCATGGCATCTCC
IHNV_OC_F5	GCTAGCGGATCTGACGGTTC
IHNV_OC_R5	TACTTTTTTTATACAGGGTCGGCATGGCATCTC
IHNV_OC_R5	TACTTTTTTTATACAGGGTCGGCATGGCATCTC
For construction of rIHNV G△TM	
CMV_enhancer_OC_F	ACATGACCTTATGGGACTTTCCTACTTG
IHNVG△TM_OC_R	GATTGAGGTCCTTTAACTCCAGTGGAGTGATTGAAGG
IHNVG_stop_F	AAAGGACCTCAATCTTCACTTCCTC
IHNV_9761_R	CTTCCAGGGGGTGTCTCCAATG
IHNV_9738_F	GACACCCCCTGGAAGCAAAG
Kan_9342_R	GTCCCATAAGGTCATGTACTGGGC
For construction of supporting plasmids	
IHNV_N_OC_F	GACTCATTCCGCTGAGCTCGAGTCTAGAGGGCCC
pc_ori_3888_R	GGTTTCGCCACCTCTGACTTGAG
pc_ori_3888R	CAGAGGTGGCGAAACCCG
IHNV_N_OC_R	GAGTTCGCTTGTCATCCAAGCTTAAGTTTAAACGCTAGC
IHNV_N_gene_F	ATGACAAGCGAACTCAGAGAGACG
IHNV_N_gene_R	TCAGCGGAATGAGTCGGAGTC
IHNV_P_OC_F	GAAGAAAGGTCAATAGGCTCGAGTCTAGAGGGCCC
IHNV_P_OC_R	CTTCTCCATCTGACATCCAAGCTTAAGTTTAAACGCTAGC
IHNV_P_gene_F	ATGTCAGATGGAGAAGGAGAACAGTTC
IHNV_P_gene_R	CTATTGACCTTTCTTCATGCGCTTC
IHNV_L_OC_F	CTAGGCGAACAATAGGCTCGAGTCTAGAGGGCCC
IHNV_L_OC_R	GATCGAAGAAGTCCATCCAAGCTTAAGTTTAAACGCTAG
IHNV_L_gene_F	ATGGACTTCTTCGATCTTGACATAGAAATAAAAC
IHNV_L_gene_R	CTATTGTTCGCCTAGTGGAAAGAAGCC
IHNV_G_NheI_F	GCTAGCATGGACACCCTGATCACCACTC
IHNV_G△TM_BamHI	GGATCCTTAACTCCAGTGGAGTGATTGAAGG
For construction of IHNV G gene-expressing cell line	
ampR_promoter_F	GGCACTTTTCGGGGAAATGTG
pFC_IHNVG_OC_R	GATCAGGGTGTCCATGGATCTGACGGTTCACTAAACC
T2A_F	GAGGGCAGAGGAAGTCTTCTAACATGCGG
Tvector_ori_F	CACATTTCCCCGAAAAGTGCC
IHNV_G_gene_F	ATGGACACCCTGATCACCACTC
IHNVG_stoopX_OC_R	ACTTCCTCTGCCCTCGGACCTGTTTGCCAGGTG

(Bolded nucleotides indicate restriction enzyme site)

assembled using the Overlap cloner kit (Elpisbio), then the sequence-confirmed plasmid was designated as pIHNV-wild (Fig. 1A).

A vector containing a full IHNV genome but lacking the transmembrane and C-terminal cytoplasmic region of the G gene (pIHNV-GΔTM) was constructed using pIHNV-wild as a template. PCR primer sets (Table 1) were designed to produce three overlapping fragments that harbor GΔTM. All PCR amplified fragments were treated with *Dpn*I (Elpisbio) and were assembled using the Overlap cloner kit (Elpisbio) (Fig. 1B).

Construction of helper plasmids encoding N, P, L, and G gene

The N, P, and L genes were PCR-amplified from the pIHNV-wild vector using PCR, and each amplified product was cloned into pcDNA 3.1 (+) vector (Invitrogen) using the Overlap cloner kit. In addition, the G gene was PCR-amplified using the pIHNV-wild vector as the template, cloned into the pGEM T-easy vector, then ligated to *Nhe*I and *Bam*HI-treated pFC vector (System Bioscience). These helper plasmids were named pCMV-N, pCMV-P, pCMV-L, and pCMV-G, respectively.

IHNV G gene expressing cell line

To establish IHNV G gene-expressing EPC cells, the G gene ORF fragment was amplified using pCMV-G as a template. A fragment containing CMV promoter, T2A, and copepod green fluorescent protein (copGFP) was amplified using the Smart Nuclease All-in-one Tagged Vector (CAS-740 G, System Biosciences) as a template. The two overlapping fragments were treated with *Dpn*I (Elpisbio) and were assembled using the Overlap cloner kit (Elpisbio), then the sequence-confirmed plasmid was designated as pIHNV-G-T2A-copGFP (Fig. 1C).

EPC cells were co-transfected with the pIHNV-G-T2A-copGFP (40 ng) and the phiC31integrase vector (1960 ng, System Bioscience) using FuGENE HD reagent (Promega) according to the manufacturer's instructions. At 3 d post-transfection, cells were grown in a culture medium containing G-418 (400 μg/ml, Sigma) for selection. The selected cells were sorted into one cell in a 96-well plate using the fluorescence-activated cell sorter (BD FACS Aria III, BD Bioscience). To search cells showing the highest G gene expression among several clones, total RNA was extracted using the Hybrid-R kit (GeneAll), and cDNA was synthesized using the reverse transcription

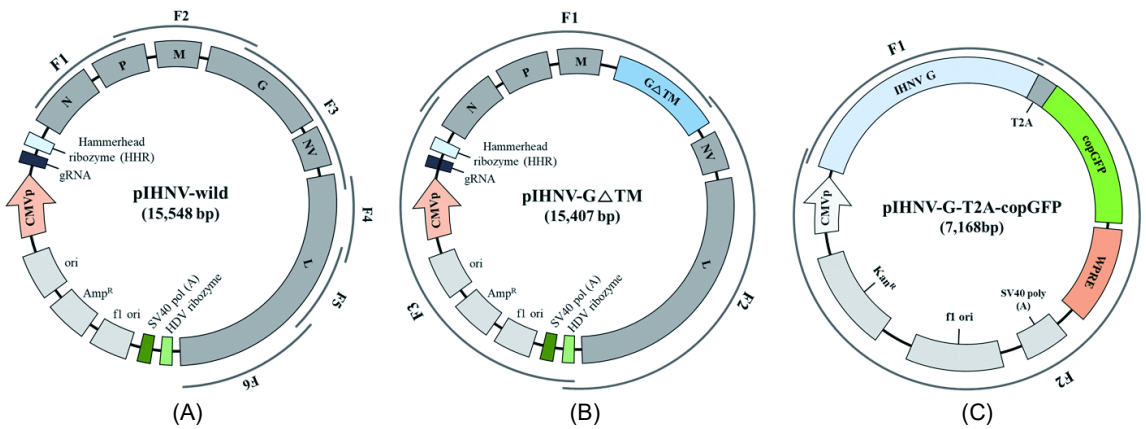

Fig. 1. Vector maps for the production of (A) a recombinant IHNV-wild (rIHNV-wild), (B) a single-cycle recombinant IHNV (rIHNV-GΔTM), and (C) IHNV G gene-expressing cells. F, fragment; gRNA, guide RNA; HDV ribozyme, Hepatitis delta virus ribozyme, copGFP, Green Fluorescent Protein from the copepod *Pontellina plumata*; T2A, WPRE,- Woodchuck hepatitis virus posttranscriptional regulatory element.

premix (Elpisbio) according to the manufacturer's instruction. Expression of the G gene was confirmed by quantitative real-time PCR (LightCycler 480, Roche) using cDNA as a template. The thermal cycling condition was 1 cycle of 15 min at 95 ℃ (pre-incubation), followed by 40 cycles of 10 sec at 95 ℃, 10 sec at 61 ℃, and 20 sec at 72 ℃. As a result of qPCR, the cell clone showing the highest G gene expression was used for recombinant single-cycle IHNV rescue.

Rescue of rIHNV-GΔTM

EPC cells expressing the IHNV G gene were co-transfected with pIHNV-GΔTM (2000 ng), pCMV-N (500 ng), pCMV-P (300 ng), pCMV-L (200 ng) and pCMV-G (500 ng; to provide another supplemental source for G protein) using FuGENE HD reagents, then were incubated in 10% FBS-L15 medium at 28 ℃ without antibiotics. After 24 hours, the medium was changed to 2% FBS-L15 supplied with 1% antibiotics (penicillin 100 U/ml, and streptomycin 100 μg/ml), then the temperature was gradually decreased to 15 ℃. When CPE appeared, cells and culture medium were freeze-thawed and cell debris was separated from the supernatant by centrifugation at 4000 g, then the separated supernatant (designated as P0) was filtered by 0.45 μm syringe filter. The P0 supernatant was inoculated into the IHNV G gene-expressing EPC cells, the stock supernatant was collected after further 2 passages in the IHNV G gene-expressing EPC cells.

RT-PCR analysis to confirm the rescue of rIHNV-GΔTM

Viral RNA was extracted from the stock supernatant using the AccuPrep Viral RNA Extraction kit (Bioneer), and cDNA was synthesized using the reverse transcription premix (Elpisbio) according to the manufacturer's instructions. RT-PCR targeting the N gene, G gene, and L gene was performed using primer sets in Table 1. Thermal cycling conditions were 1 cycle of 3 min at 95 ℃ (initial denaturation) followed by 30 cycles of 40 s at 95 ℃, 30 s at 58 ℃, and 30 s at 72 ℃, with a final extension step of 7 min at 72 ℃. PCR products were electrophoresed on 0.7% agarose gel.

Plaque assay

The stock of rIHNV-GΔTM was serially 10-fold diluted using 2% FBS-L15 supplemented with antibiotics, then inoculated into the IHNV G gene-expressing EPC cells. Each culture dish was incubated at 15 ℃ for 2 h to permeate the virus, and the inoculum was removed, then the cells were overlaid with a plaquing medium containing 0.8% agarose in 2% FBS-L15. After plaque formation was noticed under the light, it was fixed by 10% formalin for 2 h and stained with 0.2% crystal violet for 2 h at room temperature. Stained cells were rinsed with distilled water, then plaque-forming units (PFU) were counted.

Immunization of rainbow trout

Rainbow trout fingerlings (body weight 6-7 g) were obtained from a local hatchery in Korea. After 2 weeks of acclimation at 14 ℃, fish in an experimental group (15 fish) were intramuscularly (i.m.) immunized with 1.5×10^2 PFU/fish of rIHNV-GΔTM. Fish in a control group (15 fish) were injected with 50 μl of L-15 medium. At 3 weeks post-immunization, 5 fish were randomly sampled for blood collection, and the remaining fish were i.m. challenged with 2.0×10^6 PFU/fish of wild-type IHNV. Survival kinetic data were analyzed by Kaplan-Meier survival analysis (GraphPad Prism, GraphPad software), and the Log-rank test (Mantel-Cox) was used to evaluate the statistical significance ($p < 0.05$).

ELISA

A 96-well microplate (Corning) was coated with ultra-centrifuged IHNV at 1×10^6 pfu/well/100 μl of coating buffer (Na2CO3-NaHCO3 0.1 M, pH 9.6),

then was incubated overnight at 4 ℃. After 3 times washing using PBS containing 0.5% Tween-20 (PBS-T), the wells were blocked with 200 μl of blocking buffer (PBS containing 1% BSA), then were incubated for 1 h at 37 ℃. After 3 times washing, 100 μl of 10-fold diluted rainbow trout serum was added and incubated for overnight at 4 ℃. The plate was washed 3 times by washing buffer, then 100 μl of 1:100 diluted anti-Mouse rainbow trout Ig(H) monoclonal antibody (Mybiosource) in antibody dilution buffer (PBS containing 0.1% BSA) was added and incubated for 1 h at room temperature. The plate was washed three times with PBS-T, and 100 μl of 1:1000 diluted goat anti-mouse Ig-AP (Santacruz) was added and incubated for 1 h at room temperature. After three times washing with PBS-T, 200 μl of 4-nitrophenyl phosphate disodium salt hexahydrate (1 mg/ml pNPP, Sigma) was added and incubated for 30 min in the dark at room temperature, then the reaction was stopped by adding stop solution (3M NaOH). Absorbance was measured at 405 nm with a VICTOR3 multi-label plate reader (PerkinElmer). The statistical significance of the ELISA data was analyzed by Student's t-test, and $p<0.05$ was considered statistically significant.

Results

Rescue of rIHNV-GΔTM

Among several IHNV G gene-expressing EPC cell clones, one clone (clone number 7) showing the highest G gene expression in RT-qPCR (Fig. 2A) was used to rescue rIHNV-GΔTM. Through co-transfection of the EPC cells with pIHNV-GΔTM and helper vectors including pCMV-G, CPE could be induced. The rIHNV-GΔTM viral stock was made through further passages of the supernatant in the IHNV G gene-expressing cells, but the final titer of the viral stock was not high (3.0×10^3 PFU/ml) when being analyzed by plaque assay (Fig. 2B). No CPE and no plaque were observed when naive EPC cells were used for passages. To confirm the rescue of

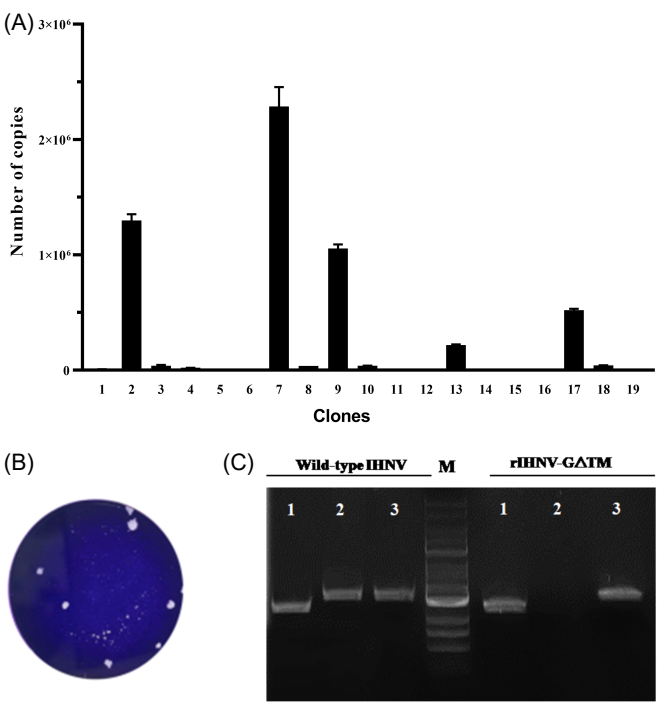

Fig. 2. (A) The RT-qPCR-based expression level of IHNV G gene in EPC cells that were transfected with pIHNV-G-T2A-copGFP and phiC31integrase vector, selected using G-418, then sorted into one cell clones. (B) Plaque assay of rIHNV-GΔTM in IHNV G gene-expressing EPC cells. (C) Verification of G gene's transmembrane and cytoplasmic region deletion in the genome of rIHNV-GΔTM by RT-PCR. lane 1, N gene; lane 2, G gene (the reverse primer matching for the cytoplasmic region of G gene); lane 3, L gene. In wild-type IHNV, all three genes were successfully amplified, but the G gene was not amplified in rIHNV-GΔTM. Marker (M), 1 kb ladder.

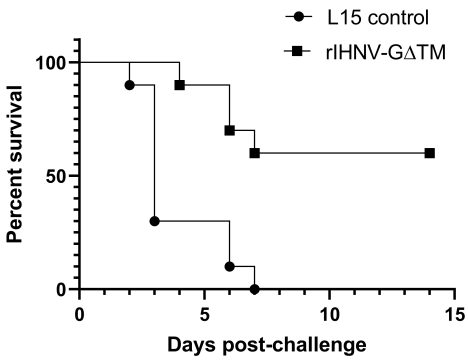

Fig. 3. Kaplan-Meier curve of survival of rainbow trout. Fish were immunized with 1.5×10^2 PFU/fish of rIHNV-GΔTM or L-15 medium alone. At 3 weeks post-immunization, fish were intramuscularly challenged with 2.0×10^6 PFU/fish of wild-type IHNV.

rIHNV-GΔTM, RT-PCR was performed, and bands corresponding to the N gene and L gene were detected from both wild-type IHNV and rIHNV-GΔTM, but a band corresponding to the G gene was not detected in rIHNV-GΔTM (Fig. 2C).

Cumulative mortality and serum ELISA titer

The group of fish immunized with rIHNV-GΔTM showed 40% cumulative mortality by the challenge with wild-type IHNV (Fig. 3). While the control group showed 100% mortality and 80% of fish in the control group died within 3 d post-challenge (Fig. 3). The survival data between the two groups were statistically significant.

Although the serum ELISA titer against IHNV in the rIHNV-GΔTM immunized group (0.56 ± 0.08) was slightly higher than that in the control group (0.43 ± 0.12), there were no significant differences between the two groups.

Discussion

In the generation of recombinant IHNVs using plasmid vectors, the T7 RNA polymerase has been frequently used to transcribe the whole genome. However, to use T7 RNA polymerase, the host cell should equip with a T7 RNA polymerase-expressing system or be infected with a vaccinia virus expressing T7 RNA polymerase (Biacchesi, 2011; Biacchesi *et al*., 2000; Kim and Kim, 2019). Ammayappan et al. (2010) rescued recombinant IHNV using the combination of CMV promoter and ribozymes (HHRz and HdvRz), which allowed the generation of recombinant IHNVs without T7 RNA polymerase expression. In this study, we also successfully rescued the G gene-truncated single-cycle IHNV (rIHNV-GΔTM) using the CMV promoter and ribozymes.

For the production of rIHNV-GΔTM, cells equipped with an IHNV full G protein-expressing system should be established, and the amount of the G protein expression can determine the titer of rIHNV-GΔTM. However, despite conducting cell cloning processes, the expression level of IHNV G protein in EPC cell clones was not high, resulting in a failure to produce a high-titer single-cycle IHNV. As the titer of rIHNV-GΔTM in the stock was low, there was no choice but to immunize fish with a low-tittered single-cycle virus (1.5×10^2 PFU/fish) in this study. However, despite the low-dose immunization, the single-cycle IHNV induced significantly higher protection in rainbow trout against IHNV infection, suggesting high immunogenicity of rIHNV-GΔTM. No significant difference in serum ELISA titers against IHNV between the rIHNV-GΔTM immunized group and the control group suggests that the immunized dose of rIHNV-GΔTM in this study might be too low to induce high humoral adaptive immune responses in rainbow trout. Although we could not analyze the adaptive cellular immune responses in this study, the superior ability of live attenuated viruses in the induction of adaptive cellular immunity has been demonstrated in mammals (Lauring *et al*., 2010; Okamura and Ebina, 2021). Moreover, the involvement of the MHC class I pathway in novirhabdoviruses infection and DNA vaccines against novirhabdoviruses has been reported (Dijkstra *et al*., 2001; Hansen and La Patra, 2002; Utke *et al*., 2007, 2008). Therefore, the

involvement of adaptive cellular immunity might be a possible way to interpret the present significantly higher protection by immunization with rIHNV-GΔTM. However, the involvement of innate immunity cannot be excluded. Further studies should be conducted to elucidate the protective mechanism of single-cycle IHNV-based vaccines.

In conclusion, the G gene-truncated single-cycle IHNV was produced through the trans-supply of IHNV G protein from cells, and the results of immunization suggest that rIHNV-GΔTM can be a good candidate for effective prophylactic vaccines. To develop cost-effective viral vaccines, the production of high-tittered viruses is essential, so more studies to increase rIHNV-GΔTM titers are required.

Acknowledgments

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