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ORIGINAL PAPER

Generation of Recombinant Viral Hemorrhagic Septicemia Virus (rVHSV) Expressing Two Foreign Proteins and Effect of Lengthened Viral Genome on Viral Growth and In Vivo Virulence

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Abstract In this study, a new recombinant VHSV (rVHSV-Arfp-Bgfp) was generated by insertion of a red fluorescent protein (RFP) gene between N and P genes, a green fluorescent protein (GFP) gene between P and M genes of VHSV genome, the expression of each heterologous gene in infected cells, and effects of the lengthened recombinant VHSV's genome on the replication ability and in vivo virulence to olive flounder (*Paralichthys olivaceus*) fingerlings were compared with previously generated rVHSVs (rVHSV-wild, rVHSV-Arfp, and rVHSV-Brfp). The expression of RFP and GFP in cells infected with rVHSV-Arfp-Bgfp was verified through fluorescent microscopy and FACS analysis. In the viral growth analysis, rVHSV-Arfp and rVHSV-Brfp showed significantly lower viral titers than rVHSV-wild, and the replication of rVHSV-Arfp-Bgfp was significantly decreased compared to that of even rVHSV-Arfp or rVHSV-Brfp. These results suggest that the genome length is a critical factor for the determination of rVHSVs replication efficiency. In the in vivo virulence experiment, the cumulative mortalities of olive flounder fingerlings infected with each rVHSV were inversely proportional to the length of the viral genome, suggesting that decreased viral growth rate due to the lengthened viral genome is accompanied with the decrease of in vivo virulence of rVHSVs. Recombinant viruses expressing multiple foreign antigens can be used for the development of combined vaccines. However, as the present rVHSV-Arfp-Bgfp still possesses an ability to kill hosts (although very weakened), researches on the producing more attenuated viruses or propagation-deficient replicon particles are needed to solve safety-related problems.

Keywords Viral hemorrhagic septicemia virus · Reverse genetics · Two foreign proteins · Lengthened genome · Viral growth · In vivo virulence

Introduction

Viral hemorrhagic septicemia virus (VHSV) belongs to the genus *Novirhabdovirus* in the family *Rhabdoviridae*, and has a negative single-stranded RNA genome that encodes six viral proteins, the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), the non-structural protein (NV), and the RNA-dependent RNA polymerase (L) in the order 3′-N–P–M–G–NV–L-5′ [1]. It has been reported that mammalian rhabdoviruses such as viral stomatitis virus (VSV) and rabies virus (RAV) have flexibility in the lengthening of their genome by the insertion of foreign genes [2, 3]. In fish rhabdoviruses, also, recombinant VHSVs (rVHSVs) that possess lengthened genome by the insertion of a heterologous viral antigen gene such as the hirame rhabdovirus (HIRRV) glycoprotein gene [4] or the West Nile virus (WNV) envelope gene [5] were generated.

In rhabdoviruses, the promoter located at the 3′ leader region is the sole promoter for the transcription of all viral genes, and the expression levels of the viral genes are progressively diminished in downstream order [6–8]. Thus, the viral properties can be changed according to the location of the foreign gene in the viral genome. However, the

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effects of inserted foreign gene locations and lengthened viral genome on VHSV properties have been poorly investigated. Previously, we generated recombinant VHSVs (rVHSV-Arfp and rVHSV-Brfp) that contain just red fluorescent protein (RFP) gene between N and P genes and between P and M genes, respectively, and found no significant difference in the fluorescent intensity between cells infected with rVHSV-Arfp and rVHSV-Brfp [9]. In the present study, we further generated a recombinant VHSV expressing two heterologous genes by inserting a RFP gene between N and P genes, and a green fluorescent protein (GFP) gene between P and M genes of VHSV genome (rVHSV-Arfp-Bgfp). Then, the expression of each heterologous gene in infected cells and the effects of the lengthened recombinant VHSV's genome on the replication ability and in vivo pathogenicity were analyzed.

Materials and Methods

Cells and Viruses

Epithelioma papulosum cyprini (EPC) cells purchased from American Type Culture Collection (ATCC) were cultured using Leibovitz medium (L-15, Sigma) supplemented with penicillin (100 U/ml, Sigma), streptomycin (100 μg/ml, Sigma), and 10 % fetal bovine serum (FBS, Sigma). The wild-type VHSV (VHSV KJ2008) used in this study was isolated from an olive flounder that was naturally infected with VHSV and showed typical VHSV signs. The genomic template of the recombinant VHSVs used in this study was originated from the genome of VHSV KJ2008. Recombinant VHSVs (rVHSV-wild, rVHSV-Arfp, and rVHSV-Brfp) were produced from our previous studies [9, 10]. Wild-type VHSV and the recombinant VHSVs were cultured with L-15 supplemented with penicillin, streptomycin, and 2 % FBS. When extensive cytopathic effect (CPE) was observed, cells were detached, centrifuged at 4 °C with 4000×*g*, filtered the supernatant with 0.45 μm syringe filter (Advantec), and kept at −80 °C.

A Recombinant VHSV (rVHSV-Arfp-Bgfp) Generation Using Reverse Genetics

Previously constructed vector (pVHSV-A-RFP) to generate recombinant VHSV carrying a RFP gene between N and P genes (Fig. 1a-i) [9] was used to construct a vector for recombinant VHSV that expressing two heterologous genes, RFP and GFP. To insert GFP gene between P and M genes of VHSV genome, the 5′ and 3′ ends of the M gene ORF in the vector (pVHSV-A-RFP) were changed to have *Bss*HII and *Nhe*I sites, respectively (Fig. 1a-ii), by using site directed mutagenesis kit (SDM, Stratagene). The primers used for the mutagenesis are described in Table 1. The GFP gene ORF was PCR amplified using primers that have *Bss*HII (forward) and *Nhe*I (reverse) sites, respectively. The M gene ORF in the mutated vector was excised by digestion with *Bss*HII and *Nhe*I, then, the GFP gene ORF was inserted into the digested plasmid (Fig. 1a-ii), resulting in pVHSV-A-ΔM-GFP. The 3′ end of the P gene ORF in the pVHSV-A-RFP was mutated to have *Nhe*I site by SDM (Fig. 1a-iii), and named the vector as pVHSV-A-RFP-Psdm. The pVHSV-A-RFP-Psdm vector was digested with *Nhe*I and *Age*I, and then the digested fragment encoding M gene was ligated to the pVHSV-A-ΔM-GFP vector that was digested with the same enzymes (Fig. 1a-iv), resulting in pVHSV-Arfp-Bgfp.

EPC cells expressing T7 RNA polymerase (RNAP) were grown to about 80 % confluence and transfected with a mixture of pVHSV-Arfp-Bgfp (2 μg) and previously constructed support vectors, pCMV-N (500 ng), pCMV-P (300 ng), and pCMV-L (200 ng) using FuGENE HD (Promega) according to manufacturer's instructions. Transfected cells were incubated for 12 h at 28 °C, and shifted to 15 °C. When extensive cytopathic effect (CPE) was observed, the cells were submitted to two cycles of freeze-thawing and centrifuged at 4000×*g* for 10 min at 4 °C. The resulting supernatant (named P0) was used to inoculate fresh EPC cells monolayer in a T25 flask at 15 °C. At 7–10 days post-inoculation, the supernatant (P1) was harvested, filtered with a 0.45 μm syringe filter (Advantec), aliquoted, and stored at −80 °C.

RT-PCR and Plaque Assay

Production of the recombinant VHSV (rVHSV-Arfp-Bgfp) was verified by RT-PCR. Total RNA was extracted from the supernatant P1 using Trizol reagent (Invitrogen), and treated with DNaseI using Riboclear plus Kit (Gene All) according to the manufacturer's instructions. To synthesize first-strand cDNA, 1 μg of total RNA was incubated with 0.5 μl of random primer (0.5 μg/ml, Promega) at 80 °C for 5 min and further incubated at 42 °C for 60 min in reaction mixture containing 2 μl of each 10 mM dNTP mix (Takara), 0.5 μl of M-MLV reverse transcriptase (Promega), and 0.25 μl of RNase inhibitor (Promega) in a final reaction volume of 10 μl. The insertion of the RFP gene and GFP gene between N and P, and P and M, respectively, was confirmed by RT-PCR using primer sets as shown in Table 1. The PCR products were analyzed on a 1 % agarose gel containing Nucleic acid stain (Korea labtec) for visualization.

To determine the titer of rVHSV-Arfp-Bgfp, plaque assay was performed [11]. Briefly, EPC cells (1.5×10^6 cells/35 mm dish) were infected with serial dilutions (10^{-4} to 10^{-6}) of the recombinant virus for 1 h at 15 °C.

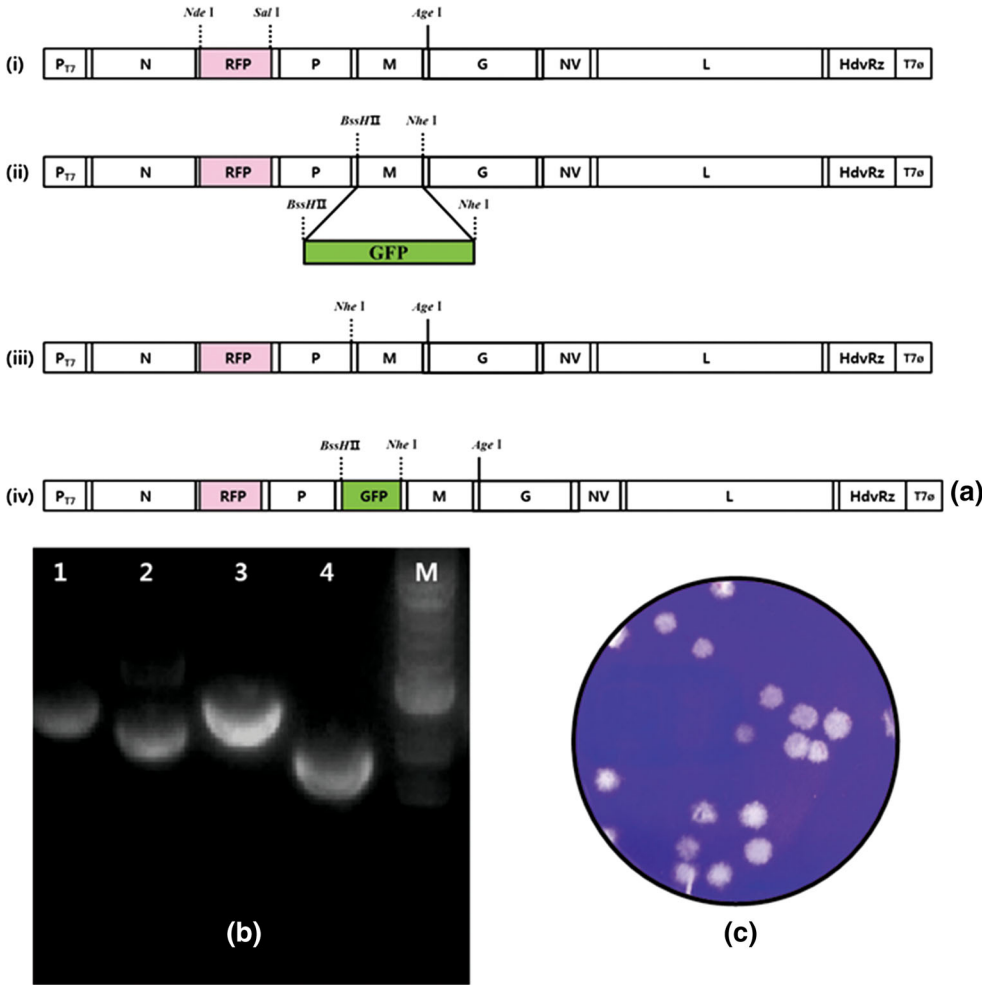

Fig. 1 **a** Schematics of the cDNA vector construction for generation of a recombinant VHSV (rVHSV-Arfp-Bgfp) harboring a *red* fluorescent protein (RFP) gene and a *green* fluorescent protein (GFP) gene between N/P and P/M junctions, respectively. (*i*) RFP gene was inserted between VHSV N and P genes (pVHSV-A-RFP vector). (*ii*) M gene was removed and GFP gene was inserted between P and G genes (pVHSV-A-ΔM-GFP vector). (*iii*) 3′ end of P gene ORF in pVHSV-A-RFP vector was mutated to have *Nhe*I site by SDM. (*iv*) GFP gene was inserted between P and M genes (pVHSV-RFP-GFP vector). P_{T7} T7 RNA polymerase promoter, *HdvRz* hepatitis delta ribozyme, *T7Φ* T7 transcription termination sequence. **b** Rescue of the recombinant virus, rVHSV-Arfp-Bgfp. Viral RNA extracted from supernatants of cells infected with rVHSV-Arfp-Bgfp was amplified by reverse transcriptase PCR (RT-PCR). *Lane 1* Forward primer specific for N gene and reverse primer for RFP gene (1.3 kb); *Lane 2* Forward primer for RFP gene and reverse primer specific for P gene (1.1 kb); *Lane 3* Forward primer specific for P gene and reverse primer for GFP gene (1.3 kb); *Lane 4* Forward primer for GFP gene and reverse primer specific for M gene (1 kb). **c** Plaque formation in Epithelioma papulosum cyprini (EPC) infected with 10^{-4} diluted rVHSV-Arfp-Bgfp stock. Cells were cultured under 0.7 % agarose containing plaquing medium, fixed at 7 days post-infection, and stained with crystal *violet* (Color figure online)

Thereafter, the inoculum was removed and the cells were overlaid with plaquing medium (0.7 % agarose in L-15 containing 2 % FBS, penicillin 100 U/ml, and streptomycin 100 μg/ml). After 7 days of incubation to allow plaque formation, the cells were fixed by 10 % formalin and stained with 5 % crystal violet for 30 min at room temperature. After rinsing of the cells with distilled water, the plaque-forming units (PFU) were counted.

Fluorescence Analysis

EPC cells (1×10^6 cells/35 mm dish) were infected at a multiplicity of infection (MOI) of 0.1 with rVHSV-RFP-GFP or rVHSV-A-RFP, and incubated at 15 °C. At 4 days post-infection, the RFP and GFP expression was confirmed by AZ100 fluorescence microscope (NIKON).

Table 1 Primers used in this study

Name of primer		Sequence (5′–3′)
For Site directed mutagenesis		
P gene	F	GAGTTGGACAAGTTGGAGTA**GCTAGC**ACACAACACATCGTACAGATA
3′-*Nhe*I SDM	R	CTATCTGTACGATGTGTTGTGT**GCTAGC**TACTCCAACTTGTCCAACT
M gene	F	CACCTCAGACAATCAACCACA**GCGCGC**ATGGCTCTATTCAAAAGAAA
5′-*BssH*IISDM	R	CTTTCTTTTGAATAGAGCCAT**GCGCGC**TGTGGTTGATTGTCTGAGGT
M gene	F	CCTCTGTCCGACCCCGGTA**GCTAGC**GAAGGACTGACCCAGAGCCGAG
3′-*Nhe*I SDM	R	CTCGGCTCTGGGTCAGTCCTTC**GCTAGC**TACCGGGGTCGGACAGAGG
eGFP-F *BssH*II	F	**GCGCGC**ATGGTGAGCAAGGGCGAG
eGFP-R *Nhe*I	R	**GCTAGC**TTACTTGTACAGCTCGTCCATG
For verification of rVHSV generation by RT-PCR		
VHSV seq801-N	F	CCATGGGGGCGTTGAGGCTCAATG
RFP-R	R	CTACAGGAACAGGTGGTGGCGG
RFP seq341	F	GCAGGACGGCTGCTTCATCTACAAGG
VHSV P ORF	R	CTACTCCAACTTGTCCAACTCC
VHSV seq1601-P	F	TCCTCAGGGTCCTCCAAACAGAAG
eGFP-R	R	TTACTTGTACAGCTCGTCCATG
eGFP seq401	F	AGGAGGACGGCAACATCCTGGGGCACA
VHSV M ORF	R	CTACCGGGGTCGGACAGAGGG

Bolded nucleotides indicate restriction enzyme sites

FACS Analysis

EPC cells (3×10^6 cells/35 mm dish) were infected with rVHSV-wild, rVHSV-Arfp, rVHSV-Brfp, or rVHSV-Arfp-Bgfp at a multiplicity of infection (MOI) of 0.1 and incubated at 15 °C. At 48 h post-infection, each single cell suspension was prepared through a 40 μm cell strainer (SPL) to remove any clumps or aggregates, washed once in phosphate-buffered saline (PBS), and analyzed by flow cytometry (BD, Accuri C6 Flow Cytometer, BD Biosciences, USA). Cells were gated (analysis of front scatter and side scatter values) to exclude cellular debris and dead cells. The RFP and GFP fluorescence were measured from 10,000 events for each sample.

Growth of rVHSVs

EPC cells in 6-well plates (3.0×10^6 cells/well) were infected with rVHSV-wild, rVHSV-Arfp, rVHSV-Brfp, or rVHSV-Arfp-Bgfp at a MOI 0.1 at 15 °C. At 1, 3, and 5 days post-infection, each supernatant was collected, and PFUs were analyzed by the plaque assay.

In Vivo Virulence of rVHSVs

Olive flounder fingerlings (4–5 g) pre-confirmed free from VHSV showing no signs of illness were infected by intramuscular (i.m.) injection with the wild-type VHSV KJ2008, rVHSV-wild, rVHSV-Arfp, rVHSV-Brfp, or rVHSV-Arfp-Bgfp (10^2, 10^3 and 10^4 PFU per fish), and control fish were injected with an equal volume of L-15 alone. Experimental fish in each group (15 fish) were kept in eighteen 30 L tanks at 14 °C and mortalities were recorded daily for 21 days post-injection.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows (Chicago, IL, USA). Data on each recombinant virus growth (plaque numbers) were analyzed by using one-way ANOVA followed by Tukey HSD post hoc test. The cumulative mortality data were analyzed by Kaplan–Meier method. $P < 0.05$ was considered statistically significant.

Results

Generation of rVHSV-Arfp-Bgfp

To produce a recombinant VHSV that can express two heterologous proteins (rVHSV-Arfp-Bgfp), ORF sequences of RFP and GFP were inserted between N and P genes, and between P and M genes, respectively, in the VHSV genome (Fig. 1a). Correct insertion of the two heterologous ORFs in the genome of the recombinant virus was verified by RT-PCR (Fig. 1b). CPE by infection with the recombinant virus was observed, and production of infectious recombinant viral particles was verified by formation of plaques (Fig. 1c).

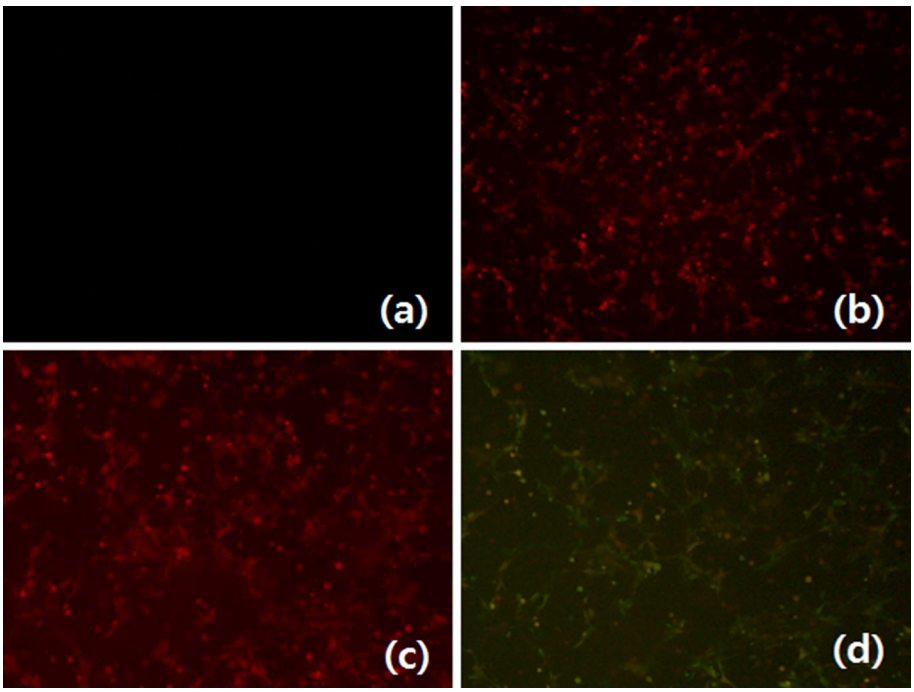

Fig. 2 Expression of red fluorescence or/and green fluorescence in EPC cells infected with rVHSV-Arfp or rVHSV-Arfp-Bgfp. **a** Mock-infected control EPC cells. **b** RFP expression in EPC cells infected with rVHSV-Arfp. **c** RFP expression in EPC cells infected with rVHSV-Arfp-Bgfp. **d** GFP expression in EPC cells infected with rVHSV-Arfp-Bgfp

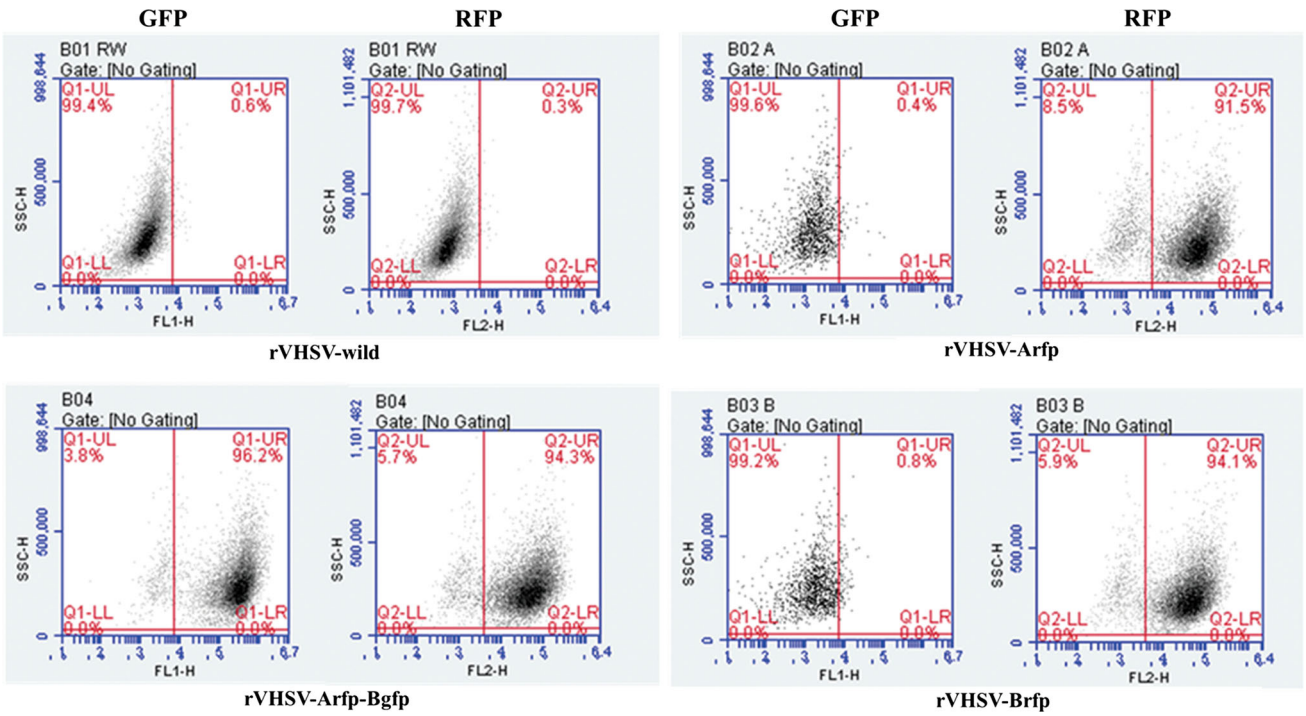

Fig. 3 The proportion of red fluorescent (RFP) or/and green fluorescent protein (GFP)-expressing cells infected with rVHSV-wild, rVHSV-Arfp, rVHSV-Brfp, or rVHSV-Arfp-Bgfp, measured by fluorescence-activated cell sorting (FACS). The cells were prepared in a single cell suspension at a concentration of 1×10^6 cells/ml, and analyzed by flow cytometry (BD Accuri C6 Flow Cytometer, BD Biosciences, USA) from 10,000 events for each sample

Fluorescence of rVHSV-RFP-GFP

Cells infected with the rVHSV-RFP-GFP showed florescence corresponding to both RFP and GFP (Fig. 2).

Demonstration of RFP or/and GFP Expression by FACS Analysis

At 48 h post-infection, the proportion of RFP-expressing cells infected with rVHSV-Arfp or rVHSV-Brfp was more than 90 %. In the cells infected with rVHSV-Arfp-Bgfp, more than 90 % of cells expressed both GFP and RFP (Fig. 3).

Replication Ability of rVHSVs

The growth of rVHSV-Arfp-Bgfp in EPC cells was analyzed using plaque assay, and compared with the growth of previously generated rVHSVs including rVHSV-wild, rVHSV-Arfp, and rVHSV-Brfp. The growth of rVHSV-Arfp-Bgfp was slower than other rVHSVs and the titer of rVHSV-Arfp-Bgfp was significantly lower than that of other rVHSVs (Fig. 4).

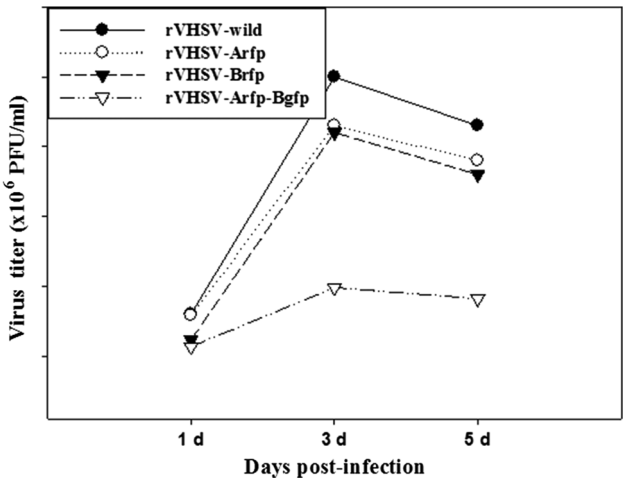

Fig. 4 Growth of rVHSV-wild, rVHSV-Arfp, rVHSV-Brfp, and rVHSV-Arfp-Bgfp. EPC cells were infected with MOI 0.1 of each virus, and the replication of viruses was analyzed by plaque assay after 1, 3, and 5 days post-infection

In Vivo Virulence of rVHSVs

The virulence of rVHSVs was analyzed by in vivo infection of olive flounder fingerlings. All fish groups administered wild-type VHSV showed 100 % cumulative mortality, while fish challenged with rVHSV-Arfp-Bgfp showed significantly lower cumulative mortalities than other rVHSVs (Fig. 5).

Discussion

In this study, we successfully produced a recombinant VHSV expressing both RFP and GFP by inserting the two genes between N–P and P–M junctions, respectively, which were verified through fluorescent microscopy and FACS analysis. Insertion of heterologous genes into viral genome inevitably leads to lengthening of genome size, which might affect on the replication efficacy of viruses especially with a small-sized RNA genome. In this study, rVHSV-Arfp and rVHSV-Brfp showed significantly lower viral titers than rVHSV-wild, and the replication of rVHSV-Arfp-Bgfp was significantly decreased compared to that of even rVHSV-Arfp or rVHSV-Brfp. These results suggest that the genome length is a critical factor for the determination of rVHSVs replication efficiency. Mebatsion et al. [2] reported that proliferation of a recombinant RAV with additional 1862 nucleotides in the genome was not different from that of standard RAV. Furthermore, An et al. [3] reported that insertion of foreign genes up to 5955 bp into vesicular stomatitis virus (VSV) genome did not affect on the replication of the recombinant virus. In Mebatsion

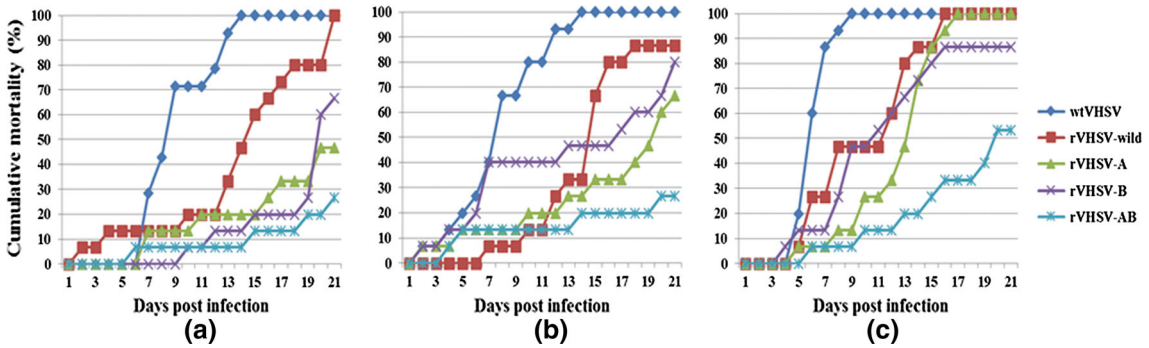

Fig. 5 Cumulative mortalities of olive flounder (*Paralichthys olivaceus*) fingerlings challenged with wild-type VHSV (wtVHSV), rVHSV-wild, rVHSV-Arfp (rVHSV-A), rVHSV-Brfp (rVHSV-B), or rVHSV-Arfp-Bgfp (rVHSV-AB) at **a** 10^2, **b** 10^3, and **c** 10^4 PFU per fish

et al. [2] and An et al. [3] experiments, the foreign gene(s) was inserted between G and L genes that are in the posterior part of viral genome. However, in this study, the foreign genes were inserted into N–P junction and P–M junction that are in the anterior part of the VHSV genome. Considering gradual weakening of expression level of rhabdoviral genes according to downstream, insertion of heterologous gene(s) in the frontal part of genome would greatly influence on the expression of the subsequent viral genes, which could act as a limiting factor for viral replication. Wertz et al. [12] and Nzonza et al. [5] also reported that insertion of a heterologous gene between N and P genes of VSV or VHSV genome resulted in lowering of the recombinant viral titers. Although insertion of foreign genes in the hind region of VHSV genome might be a way to get high-titer recombinant VHSVs, previously we had demonstrated that insertion of a foreign gene even between M and G genes of VHSV genome led to great decrease of expression level of the foreign gene [9].

In the present in vivo virulence experiment, the cumulative mortalities of olive flounder fingerlings infected with each rVHSV were inversely proportional to the length of the viral genome, suggesting that decreased viral growth rate due to the lengthened viral genome is accompanied with the decrease of in vivo virulence of rVHSVs. Recombinant viruses expressing multiple foreign antigens can be used for the development of combined vaccines. However, as the present rVHSV-Arfp-Bgfp still possesses an ability to kill hosts (although very weakened), researches on the producing more attenuated viruses or propagation-deficient replicon particles are needed to solve safety-related problems.

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