한국어병학회지 제33권 제2호 (2020)
J. Fish Pathol., 33(2) : 163~169

www.ksfp.org
pISSN 1226-0819, eISSN 2233-5412
http://dx.doi.org/10.7847/jfp.2020.33.2.163

Generation of heterologous proteins-expressing recombinant snakehead rhabdoviruses (rSHRVs) using reverse genetics

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Snakehead rhabdovirus (SHRV) is different from other fish novirhabdoviruses such as viral hemorrhagic septicemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), and hirame rhabdovirus (HIRRV) in that it replicates at high temperatures. Therefore, the delivery of foreign proteins to fish living at high water temperature would be possible by using recombinant SHRVs. In the present study, to evaluate the possible use of SHRV as a vehicle for foreign proteins delivery, we generated a recombinant SHRV that contains an enhanced-GFP (eGFP) gene between nucleoprotein (N) and phosphoprotein (P) genes (rSHRV-A-eGFP), and another recombinant SHRV expressing two heterologous genes by inserting an eGFP gene between N and P genes, and mCherry gene between P and M genes (rSHRV-AeGFP-BmCherry). Epithelioma papulosum cyprini (EPC) cells infected with the recombinant SHRVs showed strong fluorescence(s), suggesting the possible availability of recombinant SHRVs for the development of combined vaccines by expressing multiple foreign antigens.

Key words: Recombinant snakehead rhabdovirus (rSHRVs), Reverse genetics, Delivery of heterologous genes.

Introduction

Snakehead rhabdovirus (SHRV) is an enveloped, single negative-stranded RNA virus belonging to the genus *Novirhabdovirus* in the family *Rhabdoviridae* (Kasornchandra et al., 1992; Amarasinghe et al., 2019). The viral genome encodes six proteins in the order of nucleoprotein (N), polymerase-associated phosphoprotein (P), matrix protein (M), glycoprotein (G), non-virion protein (NV), and RNA-dependent RNA polymerase (L). The presence of the NV gene in the genome is a characteristics of the genus *Novirhabdovirus* (Kurath et al., 1985). SHRV was first isolated from snakehead fish (*Ophicephalus striatus*) during an epizootic ulcerative syndrome (EUS) outbreak in Thailand (Wattanavijarn et al., 1986), but the role of SHRV in EUS epizootics had not been exactly demonstrated (Kasornchandra et al., 1992). It is now well-known that infection by a fungal species, *Aphanomyces invadans*, is the primary cause of EUS (Iberahim et al., 2018; Herbert et al., 2019), and SHRV is not the principal cause of EUS.

The production of recombinant viruses using reverse genetic technology has made it possible to analyze the function of each viral gene, through which our understanding on the mechanism of virus replication and pathogenesis could be enhanced. In addition, reverse genetically rescued recombinant viruses can be utilized as live-attenuated vaccines and foreign genes delivery vehicles. Various types of recombinant novirhabdoviruses such as infectious hematopoietic

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necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) have been generated using reverse genetics, and the availability of attenuated recombinant viruses as live vaccines has also been investigated (Romero et al., 2011; Rouxel et al., 2016; Kim and Kim, 2019). In SHRV, however, only NV gene-mutated or NV gene-deleted recombinant SHRV had been rescued for the analysis of NV gene function in viral replication and virulence (Johnson et al., 2000; Alonso et al., 2004). Later, Gabor et al. (2013) mentioned that they had produced a green fluorescent protein (GFP)-expressing recombinant SHRV by replacing SHRV NV ORF with GFP ORF.

In contrast to other fish novirhabdoviruses such as VHSV, IHNV, and hirame rhabdovirus (HIRRV) that can replicate only at low temperature, SHRV replicates at high temperatures, which would be advantageous in the delivery of foreign proteins to fish that are living in high water temperatures. In the present study, to evaluate the possible use of SHRV as a vehicle for foreign proteins delivery, we generated a recombinant SHRV that contained an enhanced-GFP (eGFP) gene between N and P genes (rSHRV-A-eGFP), and another recombinant SHRV expressing two heterologous genes by inserting an eGFP gene between N and P genes, and mCherry gene between P and M genes (rSHRV-AeGFP-BmCherry).

Materials and methods

Cells and virus

Epithelioma papulosum cyprini (EPC) (ATCC: CRL-2872) cells were grown in Leibovitz medium (L-15, Sigma) supplemented with penicillin-streptomycin (Welgene) and 10% fetal bovine serum (FBS, Welgene).

SHRV (ATCC-VR1386) and recombinant SHRVs were propagated in the monolayer of EPC cells at 28℃ in the presence of 2% FBS. When the cytopathic effect (CPE) was broadly observed, the supernatant was collected following centrifugation and filtered with a 0.45 μm syringe filter (Advantec), then, kept at -80°C until use.

Generation of recombinant wild-type SHRV (rSHRV-wild)

To construct a viral vector which harboring the antigenomic cDNA of SHRV, total RNA was extracted from wild type SHRV-infected EPC cells. The antigenomic cDNA of SHRV was divided into six fragments (#1 - #6) and amplified by PCR using primers in Table 1. To exactly trim the ends of SHRV genome, hammerhead ribozyme was fused to 5' end of fragment #1, and hepatitis delta virus ribozyme was fused to 3' end of the fragment #6. All the amplified PCR fragments were cloned into T-easy vector (Promega) and sequenced. The six fragments were assembled by Overlap cloner (ELPIS, Korea) according to the manufacturer's instruction, and the constructed vector was designated as pSHRV. To construct helper vectors, SHRV's N and P genes were amplified by PCR using pSHRV as a template and cloned into T-easy vector, then, ligated to pFC vector (SystemBio) using restriction enzymes (AgeI, NotI), and designated as pFC-SHRV-N and pFC-SHRV-P. SHRV's L gene was divided into two fragments due to its long size, and named as L1 and L2. Each fragment was amplified by PCR using pSHRV as a template and cloned into T-easy vector. The L1 fragment was ligated into the pFC vector using AgeI and SpeI restriction enzymes, then, the L2 fragment was inserted just behind the L1 fragment using restriction enzymes (SpeI, NotI). The constructed vector was named as pFC-SHRV-L. All primers for construction helper vectors were represented in Table 1.

EPC cells expressing T7 RNA polymerase were seeded on 35 mm dish about 80% confluence, and were transfected with the mixture of pSHRV (2000 ng), pFC-SHRV-N (500 ng), pFC-SHRV-P (300 ng), and pFC-SHRV-L (200 ng) using Fugene HD transfection reagent (Promega). When extensive cytopathic effect (CPE) was observed, cells were lysed by a

Table 1. Primers used in this study

Primers			Sequence (5'-3')
For construction of pSHRV			
HHR		SHRV HHR F3	CGCGGGAATTCGATTACCGGT*TGATAC*CTGATGAGTCCGTGAGGACG
		SHRV HHR F2	CTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTC
		SHRV HHR F1	GAGTAAGCTCGTCGTATCAAAAAAGATGATGATACTTGGAAG
Fragment #1		SHRV Fra1 F	GTATCAAAAAAGATGATGATACTTGGAAGAAGAGAT
		SHRV Fra1 R	TGATGGCTCATCTTAAGTTGTCTAATGTACTGT
Fragment #2		SHRV Fra2 F	ACAACTTAAGATGAGCCATCAGGAAG
		SHRV Fra2 R	GGATTTTCCCATTCTTCCGAGTACTCGACAATGC
Fragment #3		SHRV Fra3 F	GAGTACTCGGAAGAATGGGAAAATCCCATATACACAACCCC
		SHRV Fra3 R	AAAATTCCATCTCCTGTCCGTTTCAG
Fragment #4		SHRV Fra4 F	GGACAGGAGATGGAATTTTTTGATCTGGACCTCGAGAT
		SHRV Fra4 R	TAGGTGATGAGGACAATGGAGGTCT
Fragment #5		SHRV Fra5 F	CCATTGTCCTCATCACCTAACCC
		SHRV Fra5 R	CCGCTACAACCTCCTCCTCC
Fragment #6		SHRV Fra6 F	AGGAGGTTGTAGCGGGGAG
		SHRV Fra6 R	GCCGGCCGTATAGAAAAAGATGATATATTTTTTTCTAGGACAAATCCAG
HDV		SHRV HDV R1	TGCCCAGCCGGCGCCAGCGAGGAGGCTGGGACCATGCCGGCCGTATAG
		SHRV HDV R2	GCGGCCGCGTCCCATTCGCCATGCCGAAGCATGTTGCCCAGCCGGCGCCAG
For construction of helper vectors			
pFC-SHRV-N		AgeI SHRV N F	ACCGGTATGGCTTTTCAGAAAGAGTTCTTTGG
		NotI SHRV N R	GCGGCCGCTTAGGCATACTTGCTGTAGTCCTGA
pFC-SHRV-P		AgeI SHRV P F	ACCGGTATGGCAGAATCGATCGAGATG
		NotI SHRV P R	GCGGCCGCTCACTTTGTGAGTTCAGCCTTCG
pFC-SHRV-L	L1	AgeI SHRV L F	CCGACTGAAACGGACAGGAACCGGTGATGGAATTT
		L_SpeI_R	GTGCTCGGGTCAACTAGTCGATAGCTCATG
	L2	L_SpeI_F	CATGAGCTATCGACTAGTTGACCCGAGCAC
		NotI SHRV L R	GCGGCCGCTCAGCGATCCCCCAACGGGA
For construction of pSHRV-AeGFP			
SHRV 1227 F			GAGATCATCAAGAGTGCTGTCCGT
SHRV NP R			CATGGTACCCCTCCTGAAGGTTCTCTTGTGTCT
eGFP OC F			TTCAGGAGGGGTACCATGGTGAGCAAGGGC
eGFP OC R			AGTTCTGTTACGCGTTTACTTGTACAGCTCGT
SHRV NP F			TAAACGCGTAACAGAACTTCCCCCCTAACGAGT
For construction of pSHRV-AeGFP-BmCherry			
SHRV PM R			CTCCTCGCCCTTGCTCACCATGCGCGCTTTGATCTCTTTTG
mCherry_OC_F			CAAAAGAGATCAAAGCGCGCATGGTGAGCAAGGGCGAGGAG
mCherry_OC_R			GTTGGGTGTGGGTGGTCCGGACTACTTGTACAGCTCGTCCATGCCGCC
SHRV PM F			GGCGGCATGGACGAGCTGTACAAGtagTCCGGACCACCCACACCCAAC
AB_SHRV_Cassette_R			CCTAGGTCAGTCCTGATCCACTGTTC

(Double underline: Hammerhead ribozyme, Italic: HHR guide sequence, Underline: Hepatitis delta virus ribozyme)

freeze-thaw, and centrifuged at 8,000 rpm for 10 min. The collected supernatant was filtered with a 0.45 μ syringe filter and passaged on EPC cells to augment the recombinant virus.

Generation of recombinant SHRVs expressing single or dual reporter proteins (rSHRV-AeGFP and rSHRV-AeGFP-BmCherry)

To use recombinant SHRVs as a foreign gene expression vehicle, vectors for the generation of single or dual reporter protein(s)-expressing SHRVs were constructed. For the rescue of single reporter protein-expressing rSHRV, eGFP gene was inserted between N and P genes using Overlap cloner, and named as pSHRV-AeGFP. For dual reporter proteins-expressing rSHRV, mCherry gene was inserted between the P and M genes of pSHRV-AeGFP using Overlap cloner, and designated as pSHRV-AeGFP-BmCherry (primers are in Table 1).

EPC cells expressing T7 RNA polymerase were co-transfected with pSHRV-AeGFP or pSHRV-AeGFPBmCherry and helper vectors (pFC-SHRV-N, pFC-SHRV-P, and pFC-SHRV-L) using FugeneHD. Supernatants containing recombinant SHRVs were stored at -80℃ before being used. To analyze the titer of generated recombinant viruses, plaque assay was performed. EPC cells (2×10^6 cells/35 mm dish) were infected for 2 h with each serially diluted viral stock (10^{-3} to 10^{-5}) at 28℃. After incubation, the medium was removed and dishes were overlaid with plaque medium (0.7% agarose in L-15 containing 2% FBS and antibiotics), and incubated until viral plaques were distinguishable under the light. The fixing and staining were performed with 10% formalin and 5% crystal violet, respectively. After rinsing with distilled water, the plaque-forming units (PFU) were counted.

Expression of reporter proteins

To verify the expression of the reporter protein from cells infected with the recombinant viruses, cells infected with rSHRV-AeGFP or rSHRV-AeGFP-BmCherry were observed using a fluorescence microscope. EPC cells (2×10^6 cells/35 mm dish) were infected for 2 h with 10^{-4} diluted viral stock at 28℃. After incubation, the medium was removed and dishes were overlaid with plaque medium, then, the expression of fluorescence was observed using a florescent microscope.

Results

Generation of recombinant SHRVs

Recombinant SHRVs expressing single reporter protein (rSHRV-AeGFP) and dual reporter proteins (rSHRV-AeGFP-BmCherry) were successfully generated by reverse genetics method (Fig. 1). Cells infected with the recombinant viruses showed CPE, and the production of infectious viral particle was verified by the formation of plaques (Fig. 2).

Fluorescence of cells infected with rSHRV-AeGFP and rSHRV-AeGFP-BmCherry

The expression of fluorescent protein in EPC cells infected with rSHRV-AeGFP or rSHRV-AeGFP-Bm Cheery was ascertained by a fluorescence microscope. Cells infected with rSHRV-A-eGFP showed a clear green fluorescence (Fig. 3). Cells infected with rSHRV-AeGFP-BmCherry showed both green and red fluorescence (Fig. 3).

Discussion

Researches on the development of recombinant VHSVs for the platform of delivering foreign antigens or cytokines have been reported (Kim and Kim, 2019). However, as VHSV can replicate only below 20℃, the foreign proteins inserted into the genome cannot be properly expressed beyond 20℃, which would act as a hurdle to be used as a delivery vehicle in fish culturing above 20℃. On the other hand, as SHRV can replicate at high temperature range (optimal at temperatures between 28-31℃) (Johnson

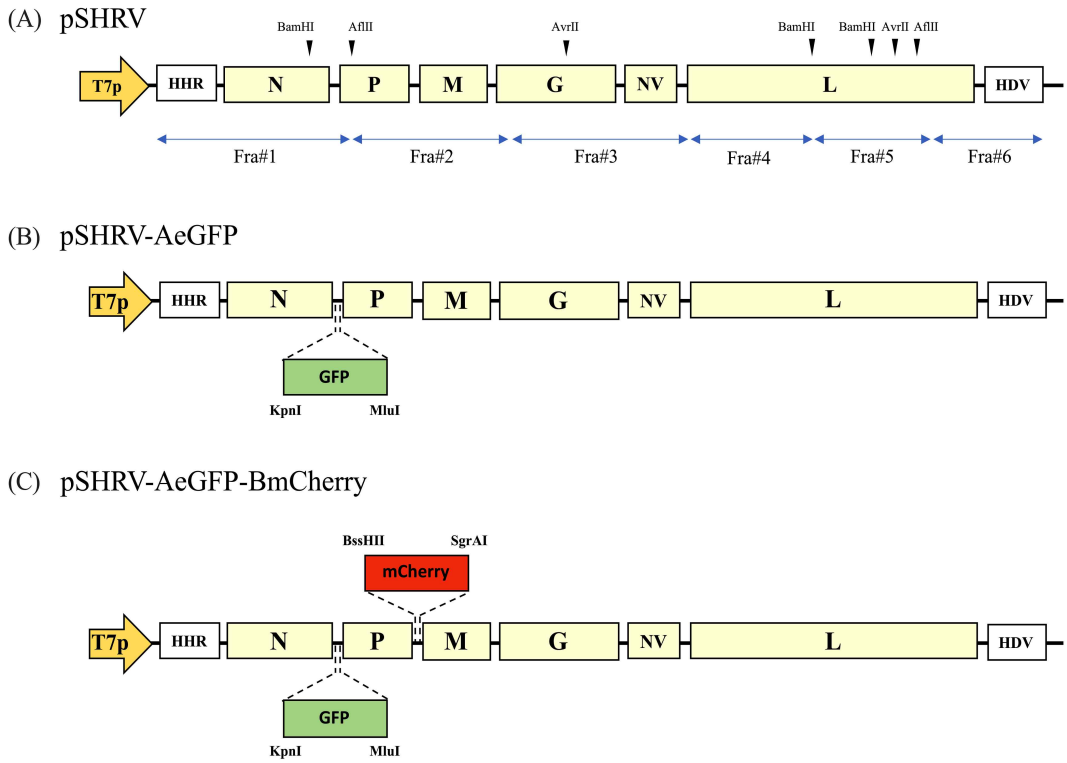

Fig. 1. Schematics representation of plasmids for the generation of a recombinant wild type SHRV (pSHRV), a rSHRV containing eGFP gene between N and P genes (pSHRV-AeGFP), a rSHRV harboring eGFP gene and mCherry gene between N/P and P/M junctions, respectively (pSHRV-AeGFP-BmCherry). T7p, T7 RNA polymerase promoter; HHR, hammerhead ribozyme; HDV, hepatitis delta virus ribozyme; T7Φ, T7 RNA polymerase terminator.

et al., 2000), it can be possible to deliver foreign proteins to fish living at high water temperature by recombinant SHRVs. In the present study, we successfully generated foreign protein(s)-expressing recombinant SHRVs, and verified the expression of the foreign protein(s) in cells infected with the recombinant SHRVs, suggesting the high potential of recombinant SHRVs for the development of combined vaccines by expressing multiple foreign antigens. Previously, Kim et al. (2016) reported that rVHSV containing eGFP and red fluorescent protein (RFP) ORFs in the genome showed retarded growth and severely weakened virulence in olive flounder (*Paralichthys olivaceus*). Similarly, in the growth of recombinant SHRVs, rSHRV-AeGFP showed significantly lower viral titer than rSHRV-wild, and the plaque size of rSHRV-AeGFP-BmCherry was significantly smaller than rSHRV-AeGFP. These results suggest that a lengthened genome size by the insertion of foreign genes into novirhabdoviral genome can lead to viral attenuation. Phelan et al. (2005) reported that more than 40% mortalities were occurred by the infection of adult zebrafish (*Danio rerio*) with SHRV. However, in our preliminary experiments, we could not get any mortalities by the infection with SHRV in zebrafish and even in fingerlings of snakehead fish (*Channa argus*). As the present recombinant SHRVs also did not induce any mortality in zebrafish, we could not demonstrate in vivo attenuation of rSHRV-AeGFP and rSHRV-A-eGFP-BmCherry. Further studies are required to know what factors are involved in the in vivo virulence of SHRV.

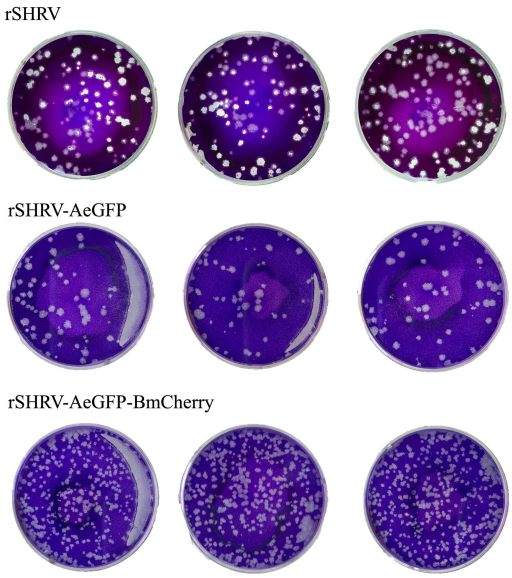

Fig. 2. Plaque formation in EPC cells infected with rSHRV (10^{-4} diluted stock), rSHRV-AeGFP (10^{-4} diluted stock), and rSHRV-AeGFP-BmCherry (10^{-3} diluted stock). Cells were cultured under 0.7% agarose containing plaque medium, fixed and stained at 3 days post-infection.

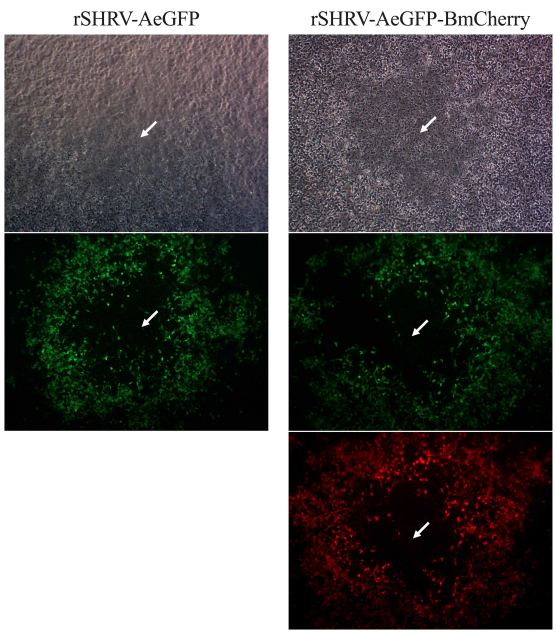

Fig. 3. Green fluorescence and green/red fluorescence in EPC cells infected with rSHRV-AeGFP (Left) and rSHRV-AeGFP-BmCherry (Right), respectively. Cells in the center region (arrow) were detached by CPE.

Acknowledgements

This work was supported by a Research Grant of Pukyong National University, Republic of Korea (2019 year).

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Manuscript Received : Aug 24, 2020
Revised : Sep 3, 2020
Accepted : Dec 1, 2020