Thesis for the Degree of Master of Science

Generation and characterization of chimeric rhabdoviruses expressing heterologous glycoproteins and Development of rapid neutralization assay using chimeric rhabdovirus

by

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August, 2020

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Advisor: Prof. Ki Hong Kim

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A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Science

in Department of Aquatic Life Medicine, The Graduate School,
Pukyong National University

August, 2020

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Contents

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요약

Rhabdovirus는 enveloped, non-segmented, negative single-stranded RNA virus로 어류에 감염되는 rhabdovirus로는 infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), snakehead rhabdovirus (SHRV), hirame rhabdovirus (HIRRV), spring viremia of carp virus (SVCV)가 있다. 이와 같은 어류 rhabdovirus는 수온이 질병의 발생에 중요한 역할을 하는 것이 특징으로, IHNV, VHSV, HIRRV, SVCV는 주로 20°C 이하의 낮은 온도에서 질병을 일으키는 반면 SHRV는 28°C에서 31°C의 높은 수온에서 질병이 발생한다. 현재까지 각각의 rhabdovirus가 가지는 증식 온도나 병독성과 같은 특성들을 결정하는데 관여하는 여러 요소들에 대해 몇몇의 연구들이 이루어져 있지만 뚜렷하게 밝혀지지 않았으며 바이러스의 종류나 strain에 따라 상반된 결과들이 보고되어 있다. 따라서 본 연구에서는 rhabdovirus를 구성하는 단백질 중 바이러스의 막 밖으로 노출되어 있어 숙주 세포에 부착, 융합함으로써 바이러스의 감염과 증식에 중요한 역할을 하는 것으로 알려진 glycoprotein (G)이 특정 온도에서 감염과 증식 그리고 병독성을 결정하는지 알아보기 위해, reverse genetics를 이용하여 다른 rhabdovirus의 G protein을 발현하는 chimeric rhabdoviruses (rSHRV-Gvhsv, rSHRV-Gsvcv, rVHSV-Gshrv, rVHSV-Gsvcv)를 제작하였다. 이를 이용하여 *in vitro*에서는 *Epithelioma papulosum cyprini* (EPC) cell에서 온도에 따른 plaque의 형성과 증식 능력을, *in vivo*에서는 zebrafish에서 병독성의 변화를 확인하고자

하였다. 그 결과, EPC cell에서 rSHRV-Gvhsv의 경우 plaque의 형성과 증식이 15°C와 20°C에서 한정되어 나타난 것을 볼 수 있었고, rVHSV-Gshrv와 rVHSV-Gsvcv는 20°C에서 가장 높은 증식 능력을 보였지만 기존의 증식 온도의 범위는 달라지지 않음을 확인할 수 있었다. 따라서 VHSV의 G protein은 VHSV가 낮은 온도에서 증식하는 특징을 결정하는데 관여하는 것으로 생각되지만, VHSV의 G protein을 높은 온도에서 잘 증식하는 바이러스인 SHRV와 SVCV로 바꾸어 주었을 경우 증식 온도의 범위가 변하지 않는 것으로 보아 VHSV의 증식 온도를 결정하는데 관여하는 요소는 G protein 이외에도 더 있을 것으로 보인다. 따라서 VHSV에서 G protein 이외의 단백질 중 낮은 온도에서의 증식을 결정하는데 관여하는 단백질이 있는지 알아보기 위해, 넓은 온도 범위에서 작동할 수 있는 SHRV의 minigenome을 제작하여 VHSV의 nucleoprotein (N), phosphoprotein (P) 그리고 RNA-dependent RNA polymerase (L) gene을 포함하는 helper plasmids와 함께 EPC cell에 transfection 하였고, SHRV의 helper plasmids를 공급해 주었을 때와 달리 15°C와 20°C에서만 형광이 나타나는 것을 확인하였다. 더 나아가, SHRV와 VHSV의 helper plasmids를 heterologous한 조합으로 공급해 주었을 경우 VHSV N과 P를 각각 공급해 주었을 때 25°C와 28°C에서 minigenome이 작동한 것으로 보아 N protein과 P protein은 낮은 온도의 증식에 직접적으로 관련이 없는 것으로 생각되어 L protein이 VHSV의 온도 결정에 관여할 가능성이 가장 높음을 확인하였다.

Zebrafish 감염 실험에서는 rVHSV-Gshrv와 rVHSV-Gsvcv가 감염성을 보이지 않았으며 rSHRV-Gvhsv는 높은 병독성을 획득하였다. 그리고 SVCV가 100%의 병독성을 나타낸 것과 다르게 rSHRV-Gsvcv는 폐사가 일어나지 않은 것을 볼 수 있었다. 따라서, SHRV와 SVCV는 G protein 보다는 다른 단백질이나 요소들이 병독성의 결정에 관여하는 것으로 보여지며, VHSV의 경우 G protein이 낮은 온도에서 감염성과 병독성을 결정하는데 중요한 역할을 하는 것으로 확인되었다.

더 나아가, rhabdovirus의 G protein이 중화 항체의 형성을 유도할 뿐만 아니라 중화 항체에 대해 인식되는 주요 단백질이라는 점을 이용하여, 제작한 chimeric rhabdoviruses 중 VHSV 보다 증식 속도가 빠른 rSHRV-Gvhsv를 기반으로 하는

VHSV에 대한 중화 항체 시험법을 개발하고자 하였다. rSHRV-Gvhsv의 사용 가능성을 평가하기 위해 토끼에서 생산한 VHSV에 대한 면역혈청을 이용하여 western blot analysis 결과, VHSV에 대한 항체가 rSHRV-Gvhsv의 G protein과 특이적으로 결합하는 것을 확인하였다. 더불어 중화 반응을 가시적으로 확인할 수 있을 뿐만 아니라 많은 개수의 시료를 한 번에 스크리닝 할 수 있도록 chimeric rhabdovirus의 nucleoprotein gene (N gene)과 phosphoprotein gene (P gene) 사이에 enhanced green fluorescent protein (eGFP) gene을 삽입하여 녹색 형광 단백질을 발현하는 chimeric rhabdovirus인 rSHRV-Gvhsv-eGFP를 제작하였고, 이를 이용해 토끼와 넙치 (*Paralichthys olivaceus*)의 면역혈청으로 중화 반응을 관찰할 수 있음을 확인하였다. 뿐만 아니라 rSHRV-Gvhsv-eGFP와 rVHSV-eGFP에 대해 중화 반응을 나타내는 성체 넙치의 혈청을 이용해 HIRRV에 대한 중화 항체 시험법을 실시하였으며, HIRRV에 대해 중화 반응이 나타나지 않는 것을 통해 VHSV의 항체에 대한 chimeric rhabdovirus의 특이성을 증명하였다.

본 연구에서 개발한 rSHRV-Gvhsv-eGFP를 이용한 VHSV의 중화 항체 시험법은 기존의 중화 항체 시험법보다 빠르고, 추가적인 과정 없이 현미경 관찰을 통해 보이는 형광의 정도로 혈청 내의 중화 항체 유무를 판단할 수 있어 편리하고, VHSV에 대한 중화 항체에 대해 특이성을 가지기 때문에 VHSV의 신속 중화 항체 시험법으로서 사용될 수 있을 것으로 여겨진다.

List of Tables

List of Figures

Chapter I.

Generation and characterization of chimeric rhabdoviruses expressing heterologous glycoproteins

Ⅰ. Introduction

Rhabdoviruses are enveloped, non-segmented, negative single-stranded RNA viruses, and some of them such as infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and spring viremia of carp virus (SVCV) are listed by the World Organization for Animal Health (OIE) since they have been the main threats in freshwater and marine fishes worldwide (Purcell et al., 2012; Mork et al., 2004). Other fish's rhabdoviruses such as snakehead rhabdovirus (SHRV) and hirame rhabdovirus (HIRRV) also have been reported in cultured snakefish and olive flounder, respectively (Kimura et al., 1986; Frerichs et al., 1989). IHNV, VHSV, SHRV, and HIRRV are classified in the genus *Novirhabdovirus* and the genome encodes 6 proteins in the order nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), non-virion protein (NV), and RNA-dependent RNA polymerase (L), whereas SVCV is included in the genus *Sprivivirus* and has no NV gene in its genome (Ahne et al., 2002; Purcell et al., 2012; Dixon and Stone, 2017).

In rhabdoviruses, the glycoprotein is the major surface antigen exposed at the viral envelope and is responsible for multiple-critical roles in viral infection and replication. The attachment to the receptor of cell membrane, the fusion of viral envelope to endosomal membrane, and the connection to matrix protein are performed by the envelope-spiked glycoprotein (Coll, 1995; Gaudin et al., 2012; Schultz, 2017). Furthermore, the G protein induces the formation of neutralizing antibodies, which constitute the main protective adaptive immunity (Flamand et al., 1993; Holland et al., 1986; Dietzschold et al., 1987).

The water temperature plays a significant role in the outbreaks of fish

rhabdoviral diseases; the occurrences of IHNV, VHSV, and HIRRV are below 20°C (Isshiki et al., 2001; Amend, 1970; Oseko et al., 1988), whereas SHRV occurs at warm water temperatures between 28°C to 31°C (Fryer, 1991). SVCV usually occurs in spring at water temperature below 20°C, while it can replicate in cell cultures up to 31°C (Ahne et al., 2002).

Biacchesi et al. (2002) reported that a reverse genetically rescued recombinant IHNV that was expressing SVCV glycoprotein or vesicular stomatitis virus (VSV) glycoprotein instead of IHNV glycoprotein (rIHNV-Gsvcv or rIHNV-Gvsv) could replicate only at temperatures between 14°C to 20°C, and suggested that other IHNV proteins would participate in the low-temperature growth of IHNV. Added to this, Alonso et al. (2004) successfully rescued a recombinant SHRV by replacing SHRV glycoprotein with IHNV glycoprotein, and described the slower replication of the recombinant virus compared to wild SHRV cultured at a temperature of 30°C. The rhabdoviral G proteins changed their cellular processing and conformations according to the temperature, and the resulting changes affected the virus attachment to cell receptors, membrane fusion and reacting with host neutralizing antibodies (Coll, 1995). The conformational changes of rhabdoviral glycoprotein depending on the temperature have been reported in IHNV and VHSV (Epsta and Coll, 1997; Cain et al., 1999). Therefore, both the glycoprotein and other viral proteins are considered to be responsible for the infectivity of rhabdoviruses at a specific temperature. Emmenegger et al. (2017) assessed the *in vivo* virulence of a chimeric rIHNV-Gsvcv to rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and koi (*Cyprinus rubrofuscus*) that were acclimated at 10°C, and found that rIHNV-Gsvcv induced a high mortality only in rainbow trout. Furthermore, koi infected with rIHNV-Gsvcv showed a high survival rate against SVCV challenge. Thus, glycoprotein exchanged chimeric fish rhabdoviruses can be used not only to investigate glycoprotein functions but

also to be utilized as prophylactic vaccines.

In the present study, we investigated the glycoprotein's functions in determination of viral replication temperature and *in vivo* virulence. To accomplish this task, several chimeric rhabdoviruses expressing other viral glycoproteins (rSHRV-Gvhsv, rSHRV-Gsvcv, rVHSV-Gshrv, and rVHSV-Gsvcv) were rescued, their characteristics in *Epithelioma papulosum cyprini* (EPC) cells were evaluated at different temperatures and their virulence in zebrafish was determined.

Ⅱ. Materials and Methods

1. Cell and viruses

Epithelioma papulosum cyprini (EPC) cells were grown in Leibovitz medium (L-15, Sigma) supplemented with 10% fetal bovine serum (FBS, Welgene) and 1% penicillin-streptomycin (Welgene) at 28°C. Recombinant SHRV, VHSV and wild-type SVCV were propagated in EPC cells in L-15 with 2% FBS and antibiotics at 15°C (VHSV) or 28°C (SHRV and SVCV) respectively. For the preparation of viral stocks, monolayer of EPC cells was infected with virus and incubated at the optimal temperature of virus. When the extensive cytopathic effect (CPE) was observed in infected cells, the supernatant was collected and centrifuged at 4,000 g for 10 min then filtered through a 0.45 ㎛ pore size filter. The obtained supernatant was aliquoted and stored at - 80°C until further use. The virus strains used in this study are described in Table 1.

2. Plasmid construction

2-1. Construction of viral plasmids

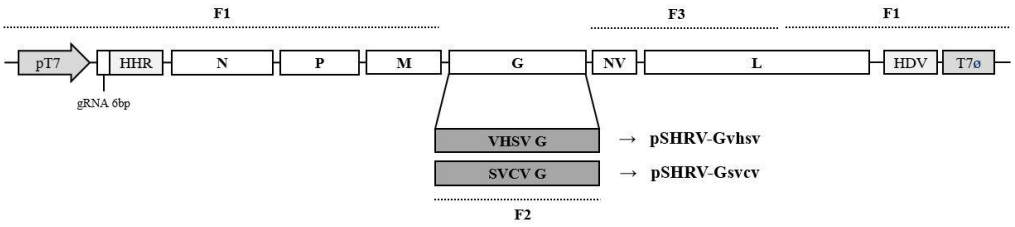
To construct four types of chimeric recombinant viral plasmids, the G gene open reading frames (ORFs) of SHRV, VHSV and SVCV were amplified by PCR using complementary DNA (cDNA) from each viral stock, then the amplified products were cloned into pGEM-T easy vector (Promega) and used as templates for constructing final form of viral plasmids. Using the vector possessing full genome of SHRV or VHSV which was previously constructed, three fragments were amplified using primer sets. The overlaps between fragments to be assembled were in the range of 15 to 20 bp. Each of amplified fragments by PCR were treated with Dpn I (Elpisbio) to eliminate non-amplified templates, then were assembled using Overlap cloner kit (Elpisbio). The reaction mixture for assembling was made in accordance with the manufacturer's protocol. The integrity of each resulting plasmid, pSHRV-Gvhsv, pSHRV-Gsvcv, pVHSV-Gshrv and pVHSV-Gsvcv, was verified by nucleotide sequencing (Macrogen Inc, Korea) (Fig 1). Primer sets used for construction of viral plasmids are listed in Table 2-1, 2-2, 2-3.

2-2. Construction of SHRV minigenome

To construct SHRV minigenome, firstly, L gene end-trailer (F1), eGFP gene (F2) and leader-N gene start (F3) were amplified respectively and assembled by overlapping PCR, then subcloned into pGEM-T easy vector to confirm the integrity of assembled fragment. After that, the overlapped fragment was amplified and inserted into a vector which has CMV promoter, hammerhead ribozyme (HHR) and hepatitis delta virus ribozyme (HDV) using Overlap cloner kit (Elpisbio). The minigenome cassette was inserted in an antisense orientation to avoid a direct production of mRNA by RNA polymerase.

After construction of minigenome plasmid, site-directed mutagenesis (SDM) was conducted using a primer set to insert the gRNA sequences (5'-TGATAC-3') in front of HHR. Primers for construction of SHRV minigenome plasmid are listed in Table 2-4.

(A)



(B)

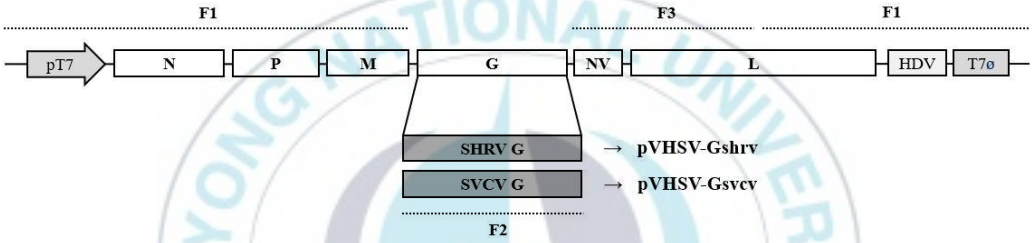


Fig 1. Schematic presentation of plasmids for the generation of recombinant chimeric SHRVs and VHSVs. All of plasmids were designed to be worked by T7 promoter and terminator. In the case of pSHRVs, hammerhead ribozyme (HHR) was added to the start of viral genome. **(A)** Plasmids for chimeric recombinant SHRV (pSHRV-Gvhsv and pSHRV-Gsvcv) **(B)** Plasmids for chimeric recombinant VHSV (pVHSV-Gshrv and pVHSV-Gsvcv)

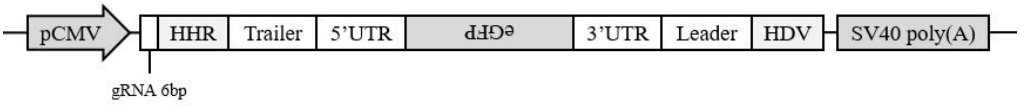


Fig 2. Structure of plasmid for SHRV minigenome. SHRV trailer-5-'UTR and 3'-UTR-leader sequences were flanked by hammerhead ribozyme (HHR) and hepatitis delta virus ribozyme (HDV), and the vector contained CMV promoter and SV40 poly(A) signal. The minigenome cassette consisting of eGFP gene, viral 3' and 5'-UTR was inserted in an antisense orientation with respect to promoter sequences for the prevention of a direct transcription into messenger RNA (mRNA) by RNA polymerase.

3. Cell transfection

3-1. Transfection and recovery of chimeric rhabdoviruses

EPC cells expressing T7 RNA polymerase were grown to about 80% confluency in 35 ㎜ dish and transfected with pSHRV-Gvhsv, pSHRV-Gsvcv, pVHSV-Gshrv or pVHSV-Gsvcv (2 ㎍) along with pCMV-N (0.5 ㎍), pCMV-P (0.3 ㎍) and pCMV-L (0.2 ㎍), using Fugene HD transfection reagent (Promega) in accordance with the manufacturer's instructions. The cells were incubated for 24 h at 28℃, and shifted to various temperatures (15, 20, 25 and 28℃). When total CPE appeared, the supernatant (P0) was obtained by freeze-thawing, centrifugation at 4,000 g for 10 min and filtering with 0.45 ㎛ syringe filter. P0 was used to be inoculated into fresh EPC cells and P1, P2 and P3 were obtained through several passages. The supernatant P4 was aliquoted and stored at -80℃ until further use.

3-2. Co-transfection of SHRV minigenome with helper plasmids

Transfection was performed in EPC cells using Fugene HD transfection reagent (Promega) as following conditions; 1; SHRV minigenome plasmid (2 ㎍) with pCMV-SHRV N (0.5 ㎍), pCMV-SHRV P (0.3 ㎍) and pCMV-SHRV L (0.2 ㎍), 2; SHRV minigenome plasmid (2 ㎍) with pCMV-VHSV N (0.5 ㎍), pCMV-VHSV P (0.3 ㎍) and pCMV-VHSV L (0.2 ㎍), 3; SHRV minigenome plasmid (2 ㎍) with pCMV-VHSV N gene (0.5 ㎍), pCMV-SHRV P (0.3 ㎍) and pCMV-SHRV L (0.2 ㎍), 4; SHRV minigenome plasmid (2 ㎍) with pCMV-VHSV P gene (0.3 ㎍), pCMV-SHRV N (0.5 ㎍) and pCMV-SHRV L

(0.2 ㎍), 5; SHRV minigenome plasmid (2 ㎍) with pCMV-VHSV L (0.2 ㎍), pCMV-SHRV N (0.5 ㎍) and pCMV-SHRV P (0.3 ㎍).

In order to confirm whether the minigenome alone produces the fluorescence or not, SHRV minigenome plasmid (3 ㎍) was transfected into cells without support of helper plasmids. After transfection, cells were incubated at 28°C for 24 h then shifted to various temperatures (15, 20, 25 and 28°C) to verify the temperature sensitivity of VHSV proteins.

4. Reverse-transcription PCR (RT-PCR) and sequencing

Generation of chimeric rhabdoviruses was verified by reverse-transcription PCR (RT-PCR). Total RNA was extracted from the aliquots of supernatant P4. To synthesize cDNA from the viral supernatant, 1 ㎍ of RNA was incubated at 70°C for 5 min, then further incubated at 37°C for 60 min and 94°C for 5 min with M-MLV reverse transcriptase containing random hexamer (Elpisbio). The N gene, M to G gene and parental G gene were amplified using primer sets (Table 2-5) and the PCR products were analysed on a 0.9% agarose gel. The amplified DNA of M to G gene was extracted by gel-purification and used to verify the replaced sequences by nucleotide sequencing (Macrogen Inc, Korea).

5. Plaque assay

Viral stocks were 10-fold serially diluted in virus growth medium (L-15 containing 2% FBS and 1% antibiotics) then inoculated into EPC cell monolayers. After 2 h of incubation at viral optimal temperature, the medium was removed and replaced with plaquing medium (0.8% agarose in L-15 containing 2% FBS and 1% antibiotics). When the plaques appeared obviously, the cells were fixed in 10% formalin for 2 h, the agarose overlay was removed, then cells were stained with 10% crystal violet at room temperature. After washing the stained cells with distilled water, the plaque-forming unit (PFU) was calculated.

6. Virus challenge in zebrafish

Adult zebrafish (*Danio rerio*) from our experimental facility were randomly divided into 7 groups (16 fish/group) and acclimated at 15°C or 20°C for 1 week. Prior to virus challenge, fish were anesthetized with 100 ppm MS-222 (Sigma) and injected with L-15 (Control group), rSHRV, rVHSV, wSVCV, rSHRV-Gvhsv, rSHRV-Gsvcv (1×10^4 PFU/20 ㎕/fish), rVHSV-Gsvcv and rVHSV-Gshrv (2×10^3 PFU/20 ㎕/fish) by intramuscular (I.M) injection. Mortalities were recorded daily for 27 days post-injection.

7. Quantitative Real-Time PCR (qRT-PCR)

To confirm the infectivity of viruses used in virus challenge, 3 fish among survived and dead fish respectively were analysed by qRT-PCR. Tissue samples from zebrafish were homogenized with Trizol (GeneAll) by Tissue Lyser II (Qiagen), then total RNA was extracted using Hybrid-R kit (GeneAll). Using 1 ㎍ of RNA, cDNA was synthesized by M-MLV reverse transcriptase containing random hexamer (Elpisbio). The resulting cDNA was used as a template for qRT-PCR. All qRT-PCR reactions were performed in a total volume of 20 ㎕ containing SYBR green PCR master mix (Enzynomics), 1 ㎕ of primer (each primer/5 pmol), 5 ㎕ of template cDNA, and 3 ㎕ of RNase free water. qRT-PCR was carried out using Light Cycler 480 (Roche) with following protocol: initial denaturation at 95°C for 15 min and 40 cycles of 95°C for 10 sec, at 60°C for 10 sec and at 72°C for 20 sec. Primer sets used in qRT-PCR analysis are listed in Table 2-6.

8. Statistical analysis

Replication ability of chimeric rhabdoviruses was analysed using SPSS for Windows (Chicago, IL, USA). Data were analysed by using one-way ANOVA followed by Turkey HSD post-hoc test.

Table 1. GenBank accession number of viruses used in this study

Virus	GenBank accession number
SHRV	AF147498.1
VHSV	JF792424.1
SVCV	MG663514.1

Table 2. Summary of primers used in this study

2-1. For amplification of G gene ORFs

Name	Sequence (5'-3')
SHRV_G_F	TCAGATCTCTACTCCCCTGCTGG
SHRV_G_R	ATGACGCTCCCAAACATGAAAC
VHSV_G_F	ATGGAATGGAATACTTTTTTCTTGGTGATTC
VHSV_G_R	TCAGACCATCTGGCTTCTGGAG
SVCV_G_F	ATGTCTATCATCAGCTACATCGCATTC
SVCV_G_R	TCAAACGAAGGACCGCATTTCGTGTGATTCTG

2-2. For construction of chimeric rSHRV plasmids

Name		Sequence (5'-3')
Fragment #1	SHRV_4500_F	CAGGGATTTCGGAGGAGGAGGT
	SHRV_G_OC_R	TGCAGCTAAGACCTTGTTCTGTTAG
Fragment #2	SHRV_Gvhsv_F	**CAAGGTCTTAGCTGCA**ATGGAATGGAATACTTTTTTCTTGGTG
	SHRV_Gvhsv_R	**GGCCTGAGTGACCGC**ATCAGACCATCTGGCTTCTGGAGAAC
	SHRV_Gsvcv_F	**CAAGGTCTTAGCTGCA**ATGTCTATCATCAG
	SHRV_Gsvcv_R	**GGCCTGAGTGACCGCA**TCAAACGAAGGAC
Fragment #3	SHRV_G_OC_F	TGCGGTCACTCAGGCCCCATCACAGTG
	SHRV_4500_R	ACCTCCTCCTCCGAAATCCCTG

(Bolded nucleotides indicate overlapped sequences)

2-3. For construction of chimeric rVHSV plasmids

Name		Sequence (5'-3')
Fragment #1	VHSV_G_OC_F	TGTGGGTTTGACTTGTTGTGTACAC
	T_easy_AmpR_R	GTTAGCTCCTTCGGTCCTCCGATCG
Fragment #2	VHSV_Gshrv_F	**ACAAGTCAAACCCACA**ATGACGCTCC CAAACATGAAAC
	VHSV_Gshrv_R	**GTTTAGGCGGCCGCGG**TCAGATCTCT ACTCCCCTGCTGG
	VHSV_Gsvcv_F	**CAAGTCAAAACCGGTA**TGTCTATCAT CAGCTACATCGC
	VHSV_Gsvcv_R	**TTTAGGCGGCCGCGGT**CAAACGAAGG ACCGCATTTCGTG
Fragment #3	T_easy_AmpR_F	AGGACCGAAGGAGCTAACCGC
	VHSV_G_OC_R	CCGCGGCCGCCTAAACCAC

(Bolded nucleotides indicate overlapped sequences)

2-4. For construction of SHRV minigenome plasmid

Name		Sequence (5'-3')
Fragment #1	SHRV_trl_OC_F	**AACGAGTAAGCTCGTC**GTATAGAAAAAGATGATAT
	SHRV_trl-eGFP_R	**GCATGGACGAGCTGTACAAGTAA**TCAGGACAAACGAGAAAGAGGTCAAC
Fragment #2	eGFP_F	ATGGTGAGCAAGGGCGAGGAG
	eGFP-SHRV_ldr_R	**GTTGACCTCTTTCTCGTTTGTCCTGA**TTACTTGTACAGCTCGTCCATGC
Fragment #3	SHRV_ldr-eGFP_F	**GCTCCTCGCCCTTGCTCACCAT**CTTGTGATTGTCTCGGTTTGATTCTTG
	SHRV_ldr-HDV_R	**TGGGACCATGCCGGCC**GTATCAAAAAAGATGATGA
HDV ribozyme_F		GGCCGGCATGGTCCCAGCC
HHR ribozyme_R		GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCA
gRNA_SDM_F		GAACCGTCAGATCCGCTAGCCTATACCTGATGAGTCCGTGAGGACG
gRNA_SDM_R		CGTCCTCACGGACTCATCAGGTATAGGCTAGCGGATCTGACGGTTC

(Bolded nucleotides indicate overlapped sequences)

2-5. For reverse-transcription PCR (RT-PCR) analysis

Name	Sequence (5'-3')
VHSV_401_F	GGAGATCTGGAGGCAAAGTGCAAG
VHSV_N_R	GCGGCCGCTTAATCAGAGTCCCCTGGGTAGTCG
VHSV_2780_F	GCGAAGGACTRCTACAATCGTGC
SVCV_G_3'_R	CCGCGGTCAAACKAARGACCGCATTTCGTG
SHRV_G_3'_R	CCGCGGTCAGATCTCTACTCCCCTGCTGG
VHSV_G_650_R	GCCTTGACCACCCTGTGATCATGTGTC
SHRV_508_F	TTGCAGCATTAGTCAAGCCCG
SHRV_N_R	GCGGCCGCTTAGGCATACTTGCTGTAGTCCTGA
SHRV_2832_F	TCCTATTCTCTGCGGAGCATGTCGG
SHRV_Gsvcv_OC_R	GGCCTGAGTGACCGCATCAAACGAAGGACCGCAT
SHRV_M_mid_F	ACACCACCCAGATCAGGAAT
VHSV_G_mid_R	TATCTTCCCAGAGGCAGTGA
SHRV_NV_R	CCTAGGTCAGTCCTGATCCACTGTTC

2-6. For quantitative Real-time PCR (qRT-PCR) analysis

Name	Sequence (5'-3')
SHRV_qPCR_F	CAGAACTTCCCCCCTAACG
SHRV_qPCR_R	CCTCCTGAAGGTTCTCTTGTG
VHSV_qPCR_F	GGCAATCAAGGCGGAGTTGGACA
VHSV_qPCR_R	GGAGAAGGTACCAGGATGGTGCGC
SVCV_qPCR_F	ATCAGGCCGATTATCCTTCCA
SVCV_qPCR_R	AGATAAGCATTCACATGCTGTAT

Ⅲ. Results

1. Generation of chimeric rhabdoviruses

To produce chimeric rhabdoviruses, EPC cells expressing T7 RNA polymerase were co-transfected with viral and supporting plasmids, and incubated at various temperatures for virus rescue since we could not predict whether the G protein affects the temperature of viral replication or not. Over several passages on fresh cells at different temperatures, we could observe a virus-induced CPE at specific temperatures. As a result, rSHRV-Gvhsv, rVHSV-Gshrv, and rVHSV-Gsvcv were successfully rescued at 15°C and 20°C, and rSHRV-Gsvcv was rescued at 25°C and 28°C. The recovery efficiency at 20°C was higher than at 15°C in rSHRV-Gvhsv, rVHSV-Gshrv and rVHSV-Gsvcv, and in the case of rSHRV-Gsvcv, it showed a higher recovery efficiency at 28°C (Data not shown).

To confirm the generation of chimeric rhabdoviruses, RT-PCR was conducted using supernatant P4. cDNA from supernatant was used as a template, and the N gene, M to exchanged G gene and the parental G gene were amplified as amplification targets. In RT-PCR analysis, the N gene and M to exchanged G gene were successfully detected whereas the parental G genes were not detected (Fig. 3). Finally, the production of recombinant viruses was verified by sequencing the amplified DNA of M to exchanged G gene.

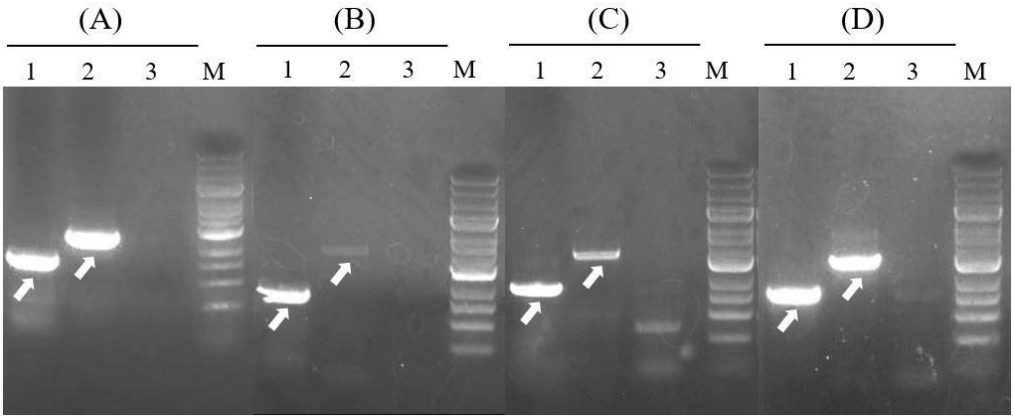


Fig 3. Confirmation of rescued chimeric rhabdoviruses by RT-PCR. **(A)** rSHRV-Gvhsv - Lane 1 : SHRV N gene (900 bp), Lane 2 : SHRV M gene to VHSV G gene (1.5 kb), Lane 3 : SHRV G gene (No band) **(B)** rSHRV-Gsvcv - Lane 1 : SHRV N gene (900 bp), Lane 2 : SHRV M gene to SVCV G gene (2 kb), Lane 3 : SHRV G gene (No band) **(C)** rVHSV-Gshrv - Lane 1 : VHSV N gene (1 kb), Lane 2 : VHSV M gene to SHRV G gene (1.7 kb), Lane 3 : VHSV G gene (No band) **(D)** rVHSV-Gsvcv - Lane 1 : VHSV N gene (1 kb), Lane 2 : VHSV M gene to SVCV G gene (1.7 kb), Lane 3 : VHSV G gene (No band). The marker is a 1kb DNA ladder (Genesta).

2. Characterization of chimeric rhabdoviruses *in vitro*

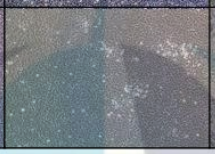
To evaluate the replication ability of chimeric rhabdoviruses at different temperatures, viruses were inoculated into EPC cells with a multiplicity of infection (MOI) of 0.0001 and supernatant was collected at 1, 3 and 5 days post-infection. To compare the replication ability with the parental virus, rSHRV and rVHSV were inoculated and the supernatant was collected together. The quantitation of virus was analysed by plaque assay.

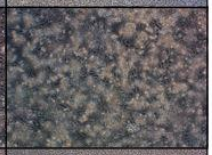
The results showed that rSHRV-Gsvcv could replicate at all temperatures conducted in this experiment, while rSHRV-Gvhsv could not replicate at 25°C and 28°C, which are the optimal temperature of rSHRV, and showed a high replication ability at 15°C and 20°C (Fig. 4-2. A). In the case of rVHSV-Gshrv and rVHSV-Gsvcv, they replicated well at low temperatures (15°C and 20°C) but could not grow at 25°C and 28°C (Fig. 4-2. B).

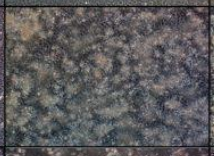
To compare the plaque morphologies of chimeric recombinant viruses at different temperatures, EPC cells were inoculated with each kind of viral stocks and incubated at different temperatures. After 7 days of incubation, cells were fixed and stained at the same time.

The plaque size of rSHRV-Gsvcv was larger than rSHRV at 20°C, 25°C and 28°C. Interestingly, rSHRV-Gvhsv formed the large plaques at 20°C whereas plaques were not observed at 25°C and 28°C (Fig. 4-3. A). rVHSV-Gshrv and rVHSV-Gsvcv showed plaques at 15°C and 20°C, and could not form any plaques at 25°C and 28°C (Fig. 4-3. B).

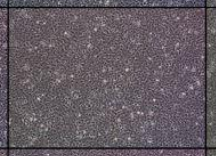
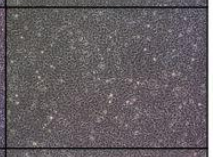
(A)

p.i. 1 day	15°C	20°C	25°C	28°C
N.I.				
rSHRV				
rSHRV-Gvhsv				
rSHRV-Gsvcv				

p.i. 3 day	15°C	20°C	25°C	28°C
N.I.				
rSHRV				
rSHRV-Gvhsv				
rSHRV-Gsvcv				

p.i. 5 day	15℃	20℃	25℃	28℃
N.I.				
rSHRV				
rSHRV-Gvhsv				
rSHRV-Gsvcv				

(B)

p.i. 1 day	15℃	20℃	25℃	28℃
N.I.				
rVHSV				
rVHSV-Gshrv				
rVHSV-Gsvcv				

p.i. 3 day	15℃	20℃	25℃	28℃
N.I.				
rVHSV				
rVHSV-Gshrv				
rVHSV-Gsvcv				

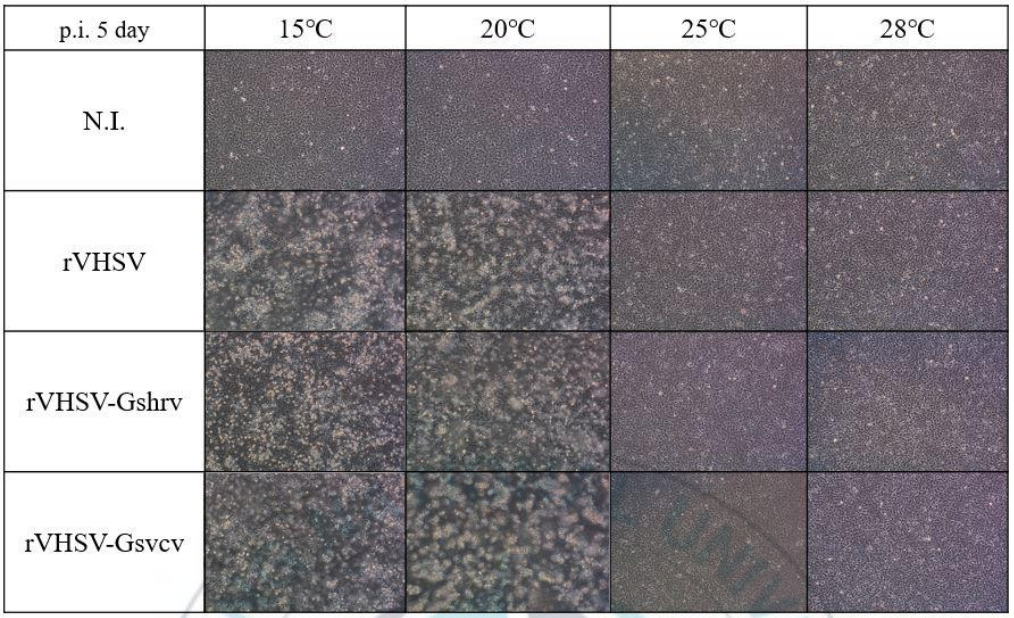
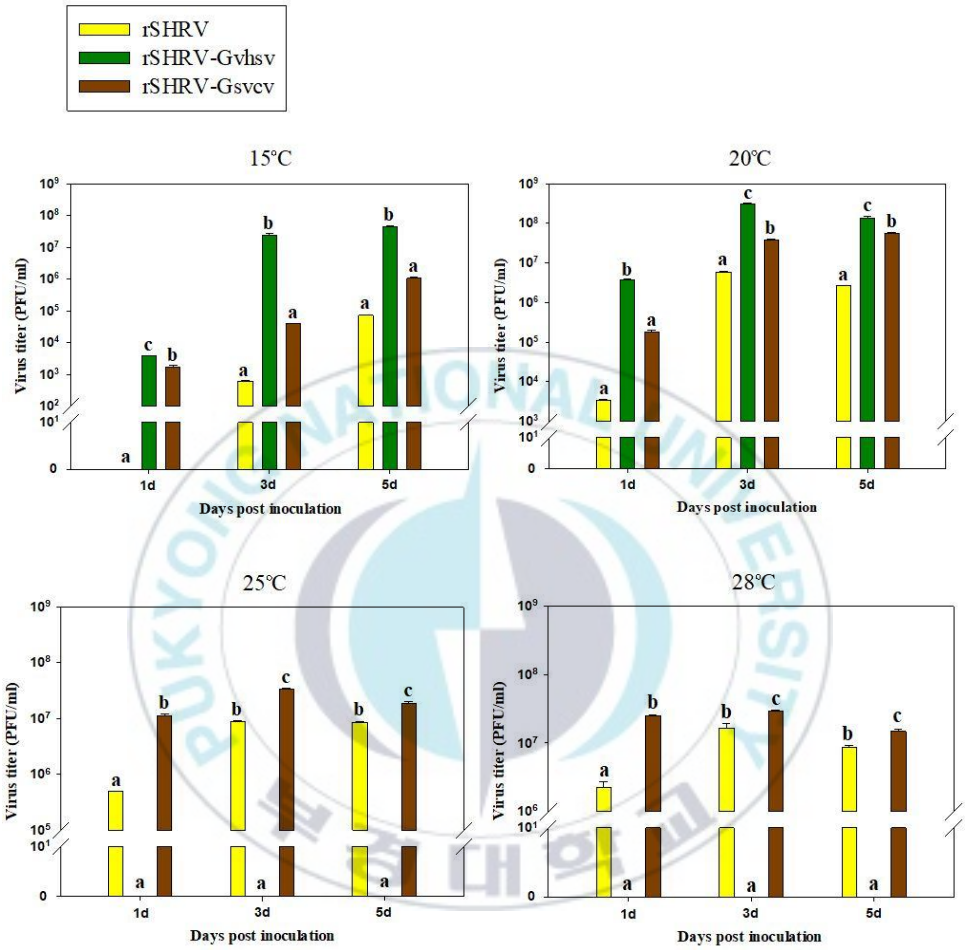


Fig 4-1. The CPE of chimeric rhabdoviruses in EPC cells at different temperatures. Cells were infected with the MOI 0.0001 and observed at 1, 3 and 5 days p.i (post-infection). N.I is non-infected cells. **(A)** CPE of rSHRV and chimeric recombinant SHRVs **(B)** CPE of rVHSV and chimeric recombinant VHSVs

(A)


rSHRV
rSHRV-Gvhsv
rSHRV-Gsvcv
15℃
20℃
25℃
28℃
Virus titer (PFU/ml)
Days post inoculation
1d
3d
5d

(B)

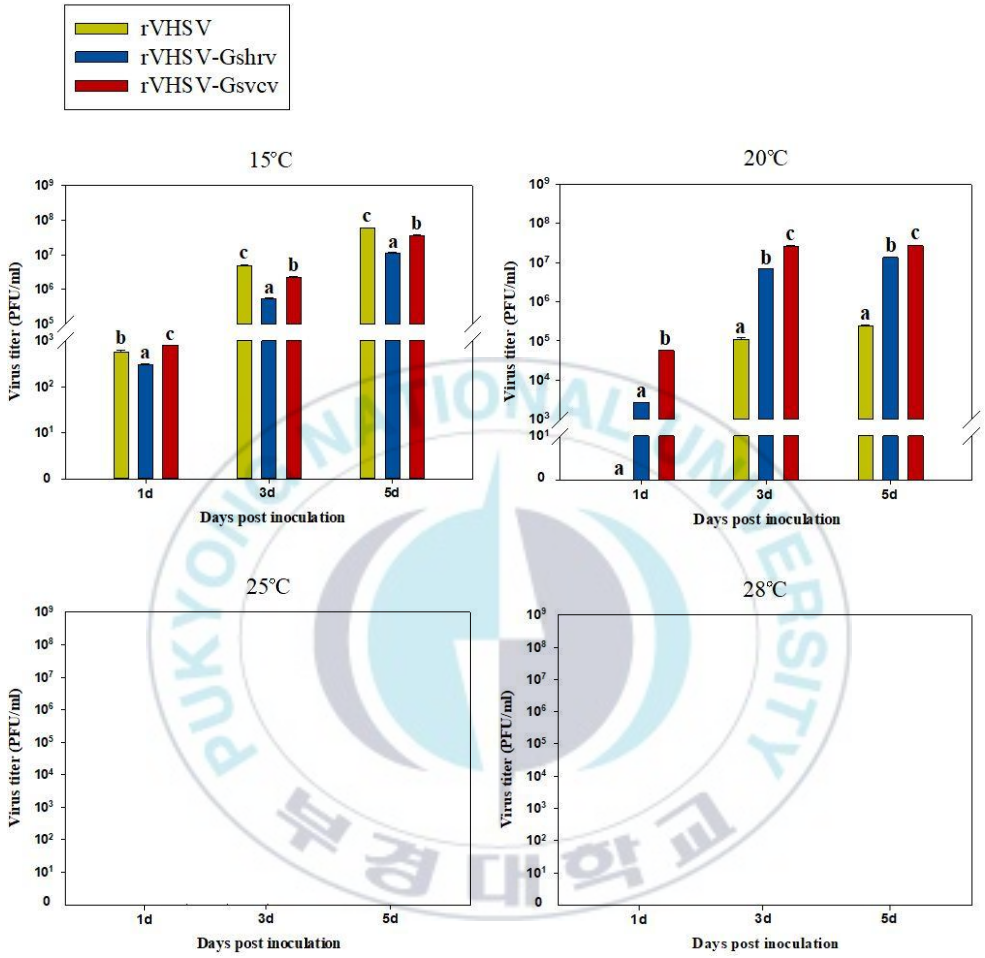
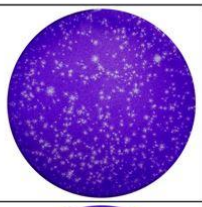
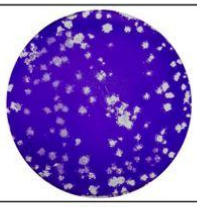
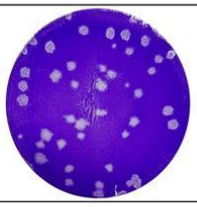
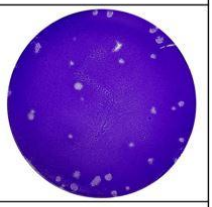
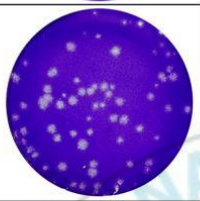
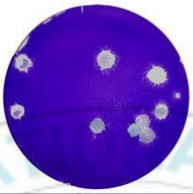
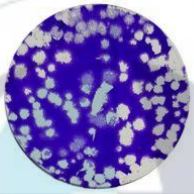
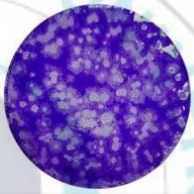
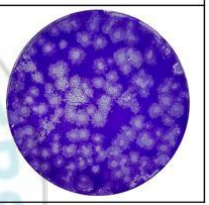


Fig 4-2. Replication ability of chimeric rhabdoviruses at different temperatures. **(A)** Replication ability of rSHRV and chimeric recombinant SHRVs at different temperatures **(B)** Replication ability of rVHSV and chimeric recombinant VHSVs at different temperatures. Different letters on the bar represent significantly different at $P < 0.05$.

(A)

	15℃	20℃	25℃	28℃
rSHRV				
rSHRV-Gvhsv			-	-
rSHRV-Gsvcv				

(B)

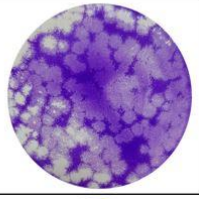
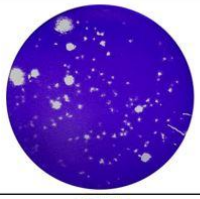
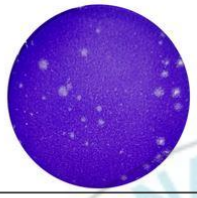
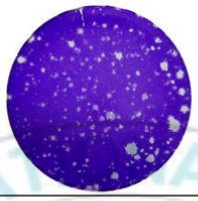
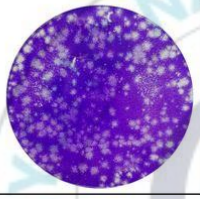
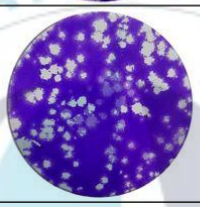
	15℃	20℃	25℃	28℃
rVHSV			-	-
rVHSV-Gshrv			-	-
rVHSV-Gsvcv			-	-

Fig 4-3. Comparison of plaque morphologies of chimeric rhabdoviruses with recombinant parental viruses at different temperatures. **(A)** rSHRV and chimeric recombinant SHRVs **(B)** rVHSV and chimeric recombinant VHSVs. (-) means that the virus did not show any plaques.

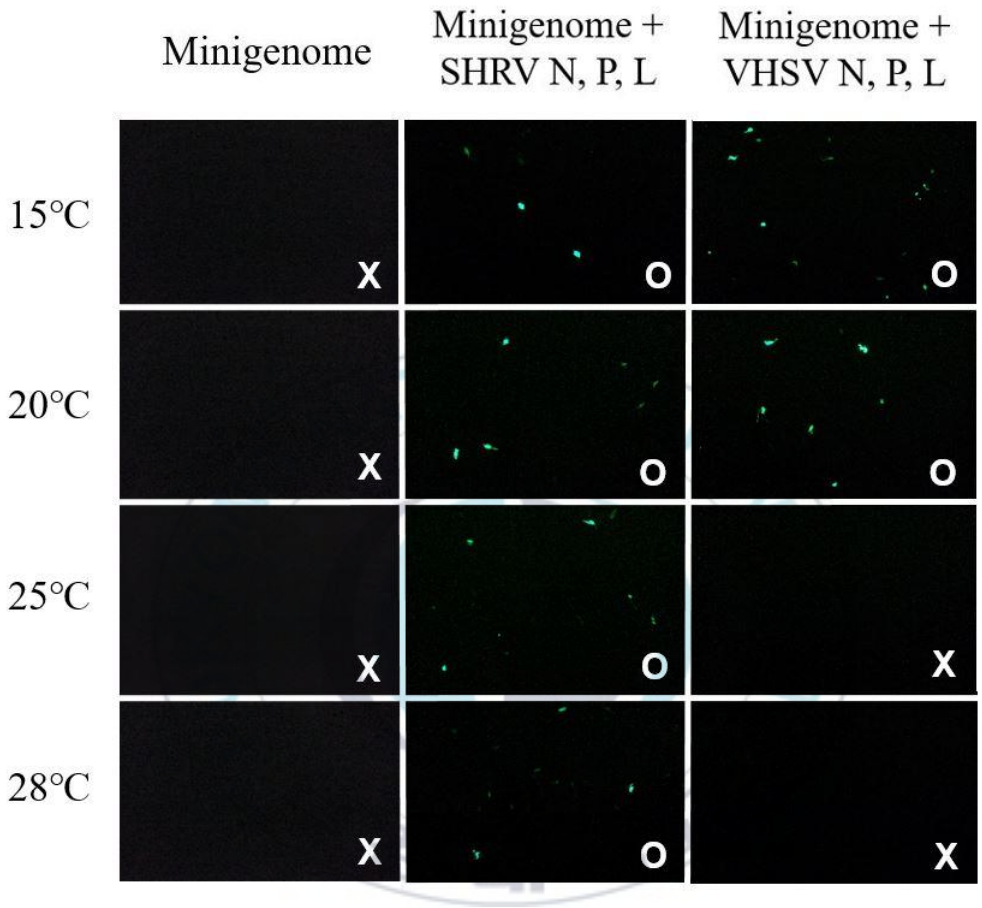
3. Investigation of determinant for temperature sensitivity using minigenome system

To investigate the presence of viral proteins associated with low-temperature sensitivity of VHSV among N, P and L protein, SHRV minigenome plasmid was constructed and transfected into EPC cells with homologous and heterologous sets of helper plasmids encoding N, P and L proteins of VHSV and SHRV.

When the SHRV helper plasmids were co-transfected with SHRV minigenome plasmid, fluorescence appeared at all temperatures conducted in this study (15, 20, 25 and 28°C). However, the minigenome was expressed only at low temperatures (15°C and 20°C) when supplied with VHSV helper plasmids (Fig. 5. A).

Additional experiment using heterologous combinations was performed to find which protein has a possibility to determine low-temperature growth. As shown in Fig. 5. B, cells transfected with VHSV N, SHRV P and SHRV L protein encoding plasmids showed fluorescence at a broad range of temperature (15, 20, 25 and 28°C), and fluorescent cells appeared at 20°C, 25°C and 28°C when transfected with a set of VHSV P, SHRV N and SHRV L protein encoding plasmids. In the case of cells supplied with a set of VHSV L, SHRV N and SHRV P proteins encoding plasmids, fluorescence was not shown at all temperatures.

(A)


Minigenome
Minigenome + SHRV N, P, L
Minigenome + VHSV N, P, L
15°C
20°C
25°C
28°C
X
O
O
X
O
O
X
O
X
X
O
X

(B)

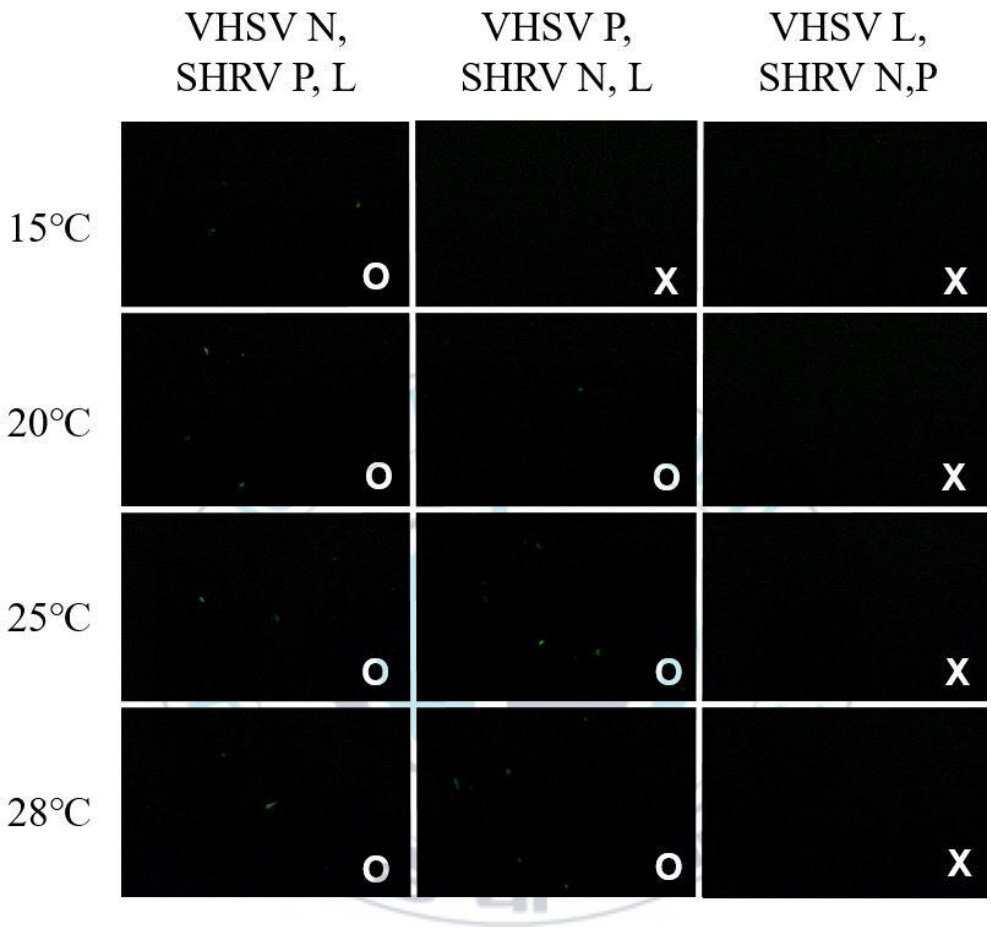


Fig 5. Fluorescence in EPC cells transfected with SHRV minigenome and various combination of homologous and heterologous helper plasmids. Cell fluorescence was photographed when the fluorescence appeared. **(A)** Cell fluorescence which was co-transfected with SHRV minigenome and homologous combinations of helper plasmids **(B)** Cell fluorescence which was co-transfected with SHRV minigenome and heterologous combinations of helper plasmids

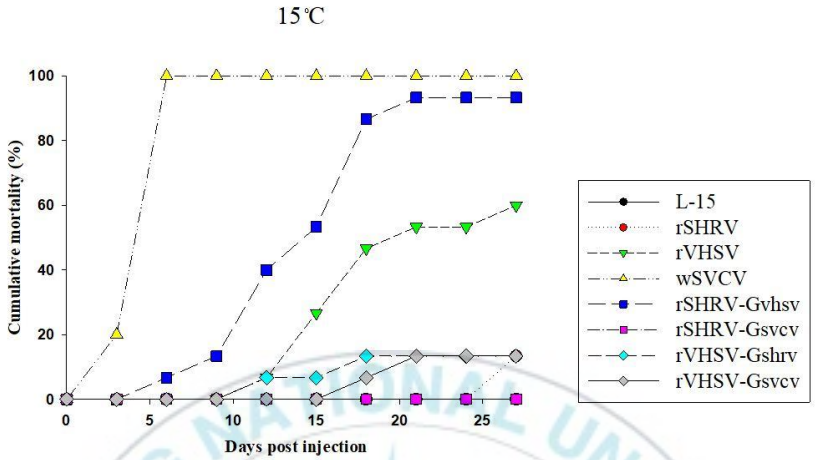
4. Characterization of chimeric rhabdoviruses *in vivo*

To verify the virulence of chimeric rhabdoviruses in zebrafish, we challenged four chimeric rhabdoviruses with rSHRV, rVHSV and wSVCV. Since there was no recombinant SVCV in our laboratory, we used wild-type SVCV instead of rSVCV. In the result of cumulative mortality, rSHRV-Gvhsv resulted in the high mortalities at 15°C (93%) and 20°C (75%) whereas remarkable mortalities did not occurred in the other chimeric viruses challenged fish. Among the glycoprotein not-exchanged-viruses, wSVCV caused 100% mortalities at 15°C and 20°C and rVHSV showed a high mortality at 15°C (60%) (Fig. 6-1).

To investigate the infectivity of chimeric rhabdoviruses in zebrafish, challenged fish were analysed by qRT-PCR. Corresponding to the result of cumulative mortality, rVHSV, wSVCV and rSHRV-Gvhsv were detected in all dead fish. In the fish that showed 13% mortalities by rSHRV (15°C), rVHSV-Gshrv (15°C and 20°C) and rVHSV-Gsvcv (15°C), the virus was not detected. For that reason, it seems that the mortalities by rSHRV, rVHSV-Gshrv and rVHSV-Gsvcv were not because of the challenged viruses.

Furthermore, we sampled 3 fish per group on the last day of challenge experiment and quantified virus. As a result, rSHRV, rVHSV, wSVCV and chimeric recombinant SHRVs indicated the infectivity in all fish. However, chimeric recombinant VHSVs were not detected. (Fig. 6-2).

(A)



(B)

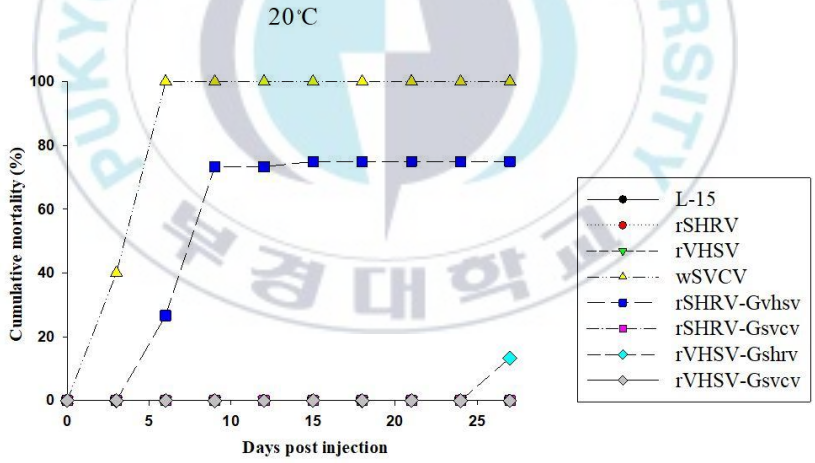
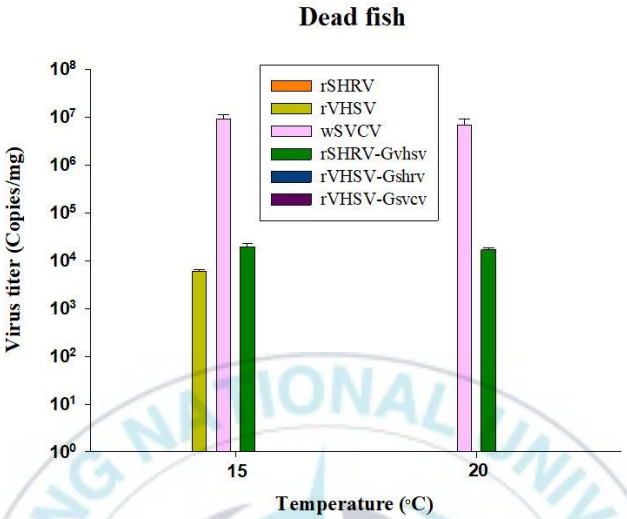


Fig 6-1. The cumulative mortality of viral challenge experiment in zebrafish. Control (L-15), parental viruses (rSHRV, rVHSV and wSVCV) and chimeric rhabdoviruses (rSHRV-Gvhsv, rSHRV-Gsvcv, rVHSV-Gshrv and rVHSV-Gsvcv) were injected by I.M injection. Mortality was monitored for 27 days. **(A)** Cumulative mortality at 15°C **(B)** Cumulative mortality at 20°C

(A)



(B)

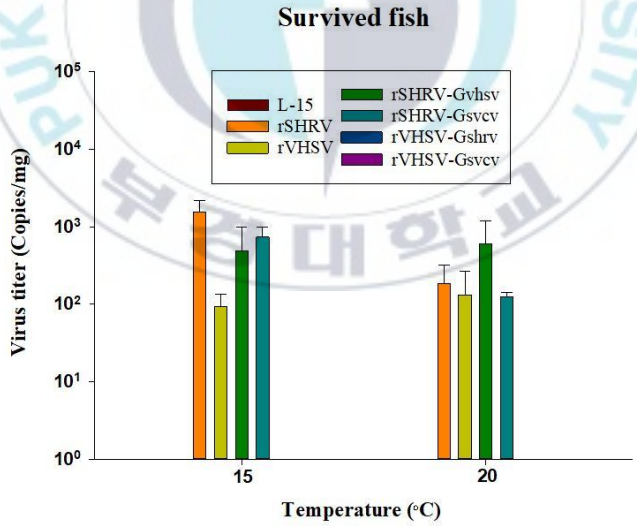


Fig 6-2. Virus titration of challenged zebrafish by qRT-PCR. The titration in dead and survived fish (3 fish/group) was analysed by qRT-PCR. **(A)** Virus titration of dead fish **(B)** Virus titration of survived fish

Ⅳ. Discussion

As the rhabdoviral glycoprotein is known as one of the factors involved in the determination of viral characteristics *in vitro* and *in vivo*, we aimed to investigate the role of fish novirhabdoviral glycoprotein at a specific temperature. To achieve this goal, we generated several kinds of chimeric rhabdoviruses expressing heterologous glycoproteins (rSHRV-Gvhsv, rSHRV-Gsvcv, rVHSV-Gshrv and rVHSV-Gsvcv) using reverse genetics and confirmed their characteristics in EPC cells and zebrafish.

In EPC cells, the replication ability and plaque morphologies of chimeric rhabdoviruses were compared to parental recombinant viruses (rSHRV and rVHSV) at various temperatures. In the case of low-temperature replication virus - VHSV, when its glycoprotein was exchanged with high-temperature replication virus's G protein - SHRV or SVCV, the replication ability of VHSV was higher than rVHSV at 20°C but the range of replication temperatures was not changed. For the replication of SHRV, the replication temperature was restricted to low temperatures when exchanged its glycoprotein with the G protein of VHSV which replicates at low temperature. This result suggests that the G protein of VHSV would participate in the determination of low-temperature growth. However, considering the result of chimeric recombinant VHSVs, it is possible that the G protein alone is not enough to determine the temperature sensitivity, suggesting that not only G protein but also other proteins or factors might be involved in the determination of low-temperature sensitivity.

In order to investigate the determinants for low temperature sensitivity among VHSV N, P and L proteins, we constructed SHRV minigenome and confirmed

its successful working at a wide range of temperatures (15, 20, 25 and 28°C) by co-transfecting helper plasmids of SHRV. However, the temperature range was restricted to low temperatures (15°C and 20°C) when co-transfected with VHSV helper plasmids, indicating that some proteins are necessarily related to viral replication temperature. From the result of additional experiment using heterologous combinations of helper plasmids of SHRV and VHSV, we could suggest that the VHSV N and VHSV P protein might not be directly responsible for low-temperature replication as cell fluorescences were shown at high temperatures when supplied with VHSV N or P protein encoding plasmids. Meanwhile, the fluorescence did not appear in cells which were co-transfected with the plasmids encoding SHRV N, SHRV P and VHSV L protein. Biacchesi et al. (2010) found that rIHNV expressing GFP could be rescued successfully with several heterologous combinations of helper plasmids of IHNV and VHSV; VHSV N, IHNV P, and IHNV L protein and VHSV N, IHNV P and IHNV L protein. Also, rVHSV expressing Tomato was rescued by supporting VHSV N, IHNV P and IHNV L protein. As for the other combinations which could not achieve the recovery of virus, the explanation for this is that the specific protein-protein interactions did not form a functional ribonucleoprotein (RNP) complex. Taken together, it means that the combination of SHRV N, SHRV P and VHSV L protein could not form the RNP complex, therefore, the results suggested that the L protein possesses high possibility to determine the low-temperature sensitivity of VHSV.

In rhabdoviruses, L protein is known to be involved in the viral replication temperature. In the studies of L protein carried out in VSV, the insertion of foreign gene into specific region of L protein and the mutation of specific amino acids induce the temperature sensitivity at high temperature (Ruedas and Perrault, 2009, Galloway and Wertz, 2009). Likewise, the L protein of VHSV interchanged into the specific temperature sensitive-strain resulted in

change of viral temperature optimum and several amino acids in L protein could determine temperature sensitivity of VHSV genotype IV (Kim et al., 2015b). Consequently, it is highly probable that G protein and L protein are involved in the determination of low-temperature replication of VHSV.

In zebrafish challenge test, we confirmed that the viral infectivity and virulence were changed depending on the heterologous glycoproteins. VHSV lost its infectivity in zebrafish when the glycoprotein was exchanged with the SHRV or SVCV G protein while SHRV gained high virulence at low temperatures when substituted its glycoprotein with VHSV G protein. Meanwhile, although SVCV caused mortalities in all zebrafish, no mortality was shown in rSHRV-Gsvcv infected fish. Taken together, these results suggest that the VHSV G protein is deeply involved in viral infectivity and virulence. Contrary to that, the SHRV and SVCV G protein might not be directly related to their virulence, suggesting that some other viral proteins or factors other than the G protein could be responsible for determination of the virulence.

To date, many studies have been performed to investigate the G protein function related to virulence in novirhabdoviruses. Among them, several studies generated chimeric recombinant viruses to verify the role of the G protein in fish virulence. Romero et al. (2005) reported that rIHNV-Gvhsv consisted of trout-virulent IHNV and VHSV strain showed similar virulence and histological lesions with IHNV rather than VHSV. Einer-Jensen et al. (2014) also reported that rIHNV which contained the G protein from low-virulent or high-virulent VHSV strain had no difference in trout-virulence. And rVHSVs exchanged the G protein between the trout-avirulent and the trout-virulent were generated and they did not show any change of the virulence compared to parental virus in trout, suggesting the VHSV G protein does not determine the trout-virulence (Yusuff et al., 2019). Interestingly, these previous reports

differ from our result in that the VHSV G protein determined the virulence in zebrafish.

In novirhabdoviruses, there are some reports that other proteins or factors than the G protein are involved in the virulence. The NV protein is well known to influence the virulence, in particular, the NV protein of VHSV and IHNV is important for viral replication and virulence in flounder and trout respectively (Kim and Kim, 2011; Kim et al., 2011; Thoulouze et al., 2004). Kim et al. (2014) found that specific amino acid of VHSV L protein induced a CPE *in vitro* whereas the G and NV protein could not. And the specific residues in 3'-UTR of VHSV modulated the virulence *in vitro* and *in vivo* (Kim et al., 2015a). Recently, Vakharia et al. (2019) suggested that VHSV N protein might contain an essential determinant for trout-virulence and could strongly enhance the virulence by P protein support.

Thus, in order to clarify the determinant for novirhabdoviral characteristics *in vitro* and *in vivo*, other viral proteins or factors, not only the G protein, should be considered, as well as the presence or not of some interactions or associations between the viral proteins in determining the replication temperature and virulence should be investigated. Moreover, it is also possible that the determinants could be located in non-coding regions, therefore, the leader, trailer or untranslated regions in the viral genome should be examined. To resolve these hypotheses, further studies including the virus of different strains or genotypes need to be studied, in addition, other viral proteins not the G protein exchanged individually or together - interspecies chimeric rhabdoviruses should be generated and compared their characteristics with the parental viruses.

In conclusion, in this study, we confirmed that the SHRV and SVCV G protein alone could not determine their virulence and the VHSV G protein had a significant role in determination of the low-temperature sensitivity and

virulence in fish. Our results expanded the G protein function in novirhabdovirus in terms of the determination of replication temperature and *in vivo* virulence. Furthermore, we verified that the L protein is likely to be responsible for the low-temperature growth of VHSV through a minigenome system.

Moreover, to utilize the chimeric rhabdoviruses generated in this study as prophylactic vaccines in multiple fish, further researches on the infectivity and virulence of chimeric rhabdoviruses in each fish species are needed in advance.

Chapter II.

Development of rapid neutralization assay using chimeric rhabdovirus

Ⅰ. Introduction

Viral hemorrhagic septicemia (VHS) is one of the serious fish diseases which cause a significant economic loss in worldwide aquaculture industry (Skall et al., 2005; Kim and Faisal, 2011). The causative agent of VHS, Viral hemorrhagic septicemia virus (VHSV), targets endothelial lining of blood vessels and causes severe hemorrhages on the skin, muscle and internal organs (Faisal et al., 2012), leading to high mortality rates up to 90% in fingerlings (Schonherz et al., 2013; Skall et al., 2005). As the virus has a wide host range, significant morbiditiy and mortality have occurred in many cultured and wild freshwater and marine fish species (Escobar et al., 2018), in particular to rainbow trout (*Oncorhynchus mykiss*) (Wolf, 1988), turbot (*Scophthalmus maximus*) (Ross et al., 1995) and Japanese flounder (*Paralichthys olivaceus*) (Isshiki et al., 2001). In Korea, VHSV is a severe threat to olive flounder (*Paralichthys olivaceus*) which is the major cultured marine fish species (Oh et al., 2009).

Currently, the presence of neutralizing antibodies (NAbs) against VHSV has been determined by conventional neutralization assay which is based on the observation of cytopathic effect (CPE) and plaque formation. In the conventional neutralization assay, serum and virus are reacted together in equal volumes and inoculated into a susceptible cell culture. If antibodies against a virus are present, the CPE or plaques in cells will not be observed since the virus is neutralized and the viral infection is inhibited. This method is accurate and specific but labor-intensive, time-consuming and the differentiation of cytopathologic changes in cell cultures is affected by operator bias (Kibenge et al., 2016). As an alternative, infected cells can be visualized by immunostaining

with virus-specific antisera or monoclonal antibodies. However, it also needs lots of labor and time (Jorgensen et al., 1991; Xue et al., 2014).

In various kinds of viruses, recombinant viruses expressing various reporter proteins (GFP, RFP and luciferase) have been used as a challenge virus in neutralization assay (Nie et al., 2016), to overcome the constraints of conventional neutralization assays. This method is more rapid and has the potential for a higher throughput compared with present neutralization assays since the neutralization activity could be observed promptly by visualization of reporter proteins in cells (Zhang et al., 2016; Deng et al., 2016; Rimmelzwaan et al., 2011; Chumbe et al., 2017; Cai et al., 2018).

In Chapter I, we confirmed that rSHRV-Gvhsv had higher replication ability compared to VHSV. Since the glycoprotein is the major surface viral protein recognized by NAbs and the NAbs are induced exclusively against the G protein (Lorenzen et al., 1990), we aimed to use the recombinant SHRV expressing VHSV G protein for a rapid neutralization assay of VHSV. In the present study, we verified that the antibodies against VHSV could bind to the G protein of rSHRV-Gvhsv. Then, to make the assay faster and high-throughput, we constructed rSHRV-Gvhsv expressing eGFP (rSHRV-Gvhsv-eGFP) and demonstrated its usefulness using various serum samples derived from rabbit and olive flounder (*Paralichthys olivaceus*).

Ⅱ. Materials and Methods

1. Cell and viruses

Epithelioma papulosum cyprini (EPC) cells were grown in Leibovitz medium (L-15, Sigma) supplemented with 10% fetal bovine serum (FBS, Welgene) and 1% penicillin-streptomycin (Welgene) at 28°C. Viruses used in this study were propagated in EPC cells in L-15 with 2% FBS and antibiotics at 15°C (wild-type VHSV-KJ2008, rVHSV-eGFP and wild-type HIRRV) or 20°C (rSHRV-Gvhsv and rSHRV-Gvhsv-eGFP) respectively. For the preparation of viral stocks, monolayer of EPC cells was infected with virus growth medium and incubated at the optimal temperatures of virus. When the extensive cytopathic effect (CPE) was observed in infected cells, the supernatant was collected and centrifuged at 4,000 g for 10 min then filtered through a 0.45 ㎛ pore size filter. The obtained supernatant was aliquoted and stored at - 80°C until further use.

2. Serum samples

2-1. Production of antisera against VHSV in rabbit

Wild-type VHSV was inoculated into EPC cells then the supernatant was collected and treated with 0.0635% formalin at 4°C for 18 h for virus inactivation. After that, virus was concentrated by ultra-centrifugation with 25% sucrose cushion at 20,000 rpm at 4°C for 1 h using Optima L-100 XP (Beckman Coulter). Then, the virus pellet was suspended with 1 ㎖ of phosphate buffered saline (PBS).

The rabbit was immunized by intraperitoneal (I.P) injection of concentrated virus and boosted through additional injections. The antisera was obtained after final injection. Injection and extraction of sera were performed by Younginfrontier (Korea).

2-2. Production of antisera against VHSV in olive flounder (*Paralichthys olivaceus*)

Olive flounder fingerlings (mean weight 22.9 g) were immunized through 2 times infection of wild-type VHSV (10^5 PFU/100 ㎕/fish) with incomplete Freund's adjuvant (Sigma). Virus was injected by intraperitoneal (I.P) injection and the water temperature was kept at room temperature. Sera were extracted at 14 days after second infection.

3. Western blot analysis

EPC cells were infected with wild-type VHSV or rSHRV-Gvhsv at the MOI of 0.01 and incubated at viral optimal temperature. When the total CPE was observed, the cells were lysed in RIPA buffer (Sigma) then denaturated by PAGESTA REDUCING SDS sample buffer (GeneAll) at 95°C for 10 min. Samples were separated by 10% SDS-PAGE gel and the proteins were transferred to nitrocellulose membrane. The membrane was blocked overnight in blocking solution (3% bovine serum albuminin TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5), and washed with TTBS (0.05% Tween 20 in TBS, pH 7.5), then incubated with polyclonal rabbit anti-VHSV serum (1:250). The membrane was washed with TTBS and incubated with an alkaline phosphatase conjugated goat anti-rabbit IgG (1:2000, Santa Cruz Biotechnology). After final washing with TTBS, bound antibodies were visualized with KPL 5-bromo-4-chloro-3-indoly phosphate and nitroblue tetrazolium (BCIP/NBT) phosphatase substrate (SeraCare).

4. Construction of viral plasmid

To construct the viral plasmid expressing reporter protein, we inserted the eGFP gene between N and P gene. Firstly, to obtain N gene-intergene-eGFP-intergene-P gene cassette, the middle of N gene to intergene (F2.a), the eGFP gene (F2.b) and the intergene to the middle of P gene (F2.c) were amplified using primer sets (Table. 3-1) and assembled by overlapping PCR, then cloned into pGEM-T easy vector (Promega) and used as a template for constructing the final form of the viral plasmid. Using pSHRV-Gvhsv previously constructed and overlapped cassette as templates, three fragments were amplified using primer sets (Table. 3-2). The overlaps between fragments to be assembled were in the range of 15 to 20 bp (Fig. 5). Each of amplified fragments by PCR was treated with Dpn I (Elpisbio) to eliminate non-amplified templates, then assembled using Overlap cloner kit (Elpisbio). The reaction mixture for assembling was made in accordance with the manufacturer's protocol. The resulting plasmid was designated as pSHRV-Gvhsv-eGFP and verified its integrity by nucleotide sequencing (Macrogen Inc, Korea).

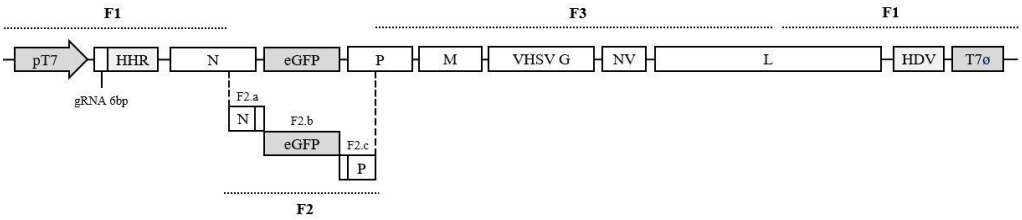


Fig 7. Construction of plasmid for rSHRV-Gvhsv-eGFP. The plasmid was designed to be worked by T7 promoter and terminator, and hammerhead ribozyme (HHR) and hepatitis delta virus ribozyme (HDV) were flanked to the leader and trailer sequence of viral genome. Fragment 2 (F2) cassette was obtained by overlapping PCR and three fragments (F1, F2 and F3) were amplified then assembled to obtain final form of viral plasmid.

5. Cell transfection and recovery of recombinant virus

EPC cells expressing T7 RNA polymerase were grown to about 80% confluency in 35 ㎜ dish and transfected with pSHRV-Gvhsv-eGFP (2 ㎍), pCMV-N (0.5 ㎍), pCMV-P (0.3 ㎍) and pCMV-L (0.2 ㎍), by using Fugene HD transfection reagent (Promega) in accordance with the manufacturer's instructions. The cells were incubated for 24 h at 28°C, after that, shifted to viral optimal temperature, 20°C. When total CPE appeared, the supernatant (P0) was obtained by freeze-thawing, centrifugation at 4,000 g for 10 min and filtering with a 0.45 ㎛ pore size filter. P0 was inoculated into fresh EPC cells and P1, P2 and P3 were obtained through several passages. The supernatant P4 was aliquoted and stored at -80°C until further use.

6. Reverse-transcription PCR (RT-PCR) and sequencing

The verification of rescued virus was analysed by reverse-transcription PCR (RT-PCR). Total RNA was extracted from the aliquots of supernatant P4. To synthesize cDNA from the viral supernatant, 1 ㎍ of RNA was incubated at 70°C for 5 min, then further incubated at 37°C for 60 min and 94°C for 5 min with M-MLV reverse transcriptase containing random hexamer (Elpisbio). The eGFP to P gene, M to VHSV G gene and SHRV G gene were amplified using

primer sets (Table. 3-3) and the products were analysed on a 0.9% agarose gel. The amplified DNA of eGFP to P gene was extracted by gel-purification and used to verify the sequences by nucleotide sequencing (Macrogen Inc, Korea).

7. Plaque assay

Viral stocks were 10-fold serially diluted in virus growth medium (L-15 containing 2% FBS and 1% antibiotics) then inoculated into EPC cell monolayers. After 2 h of incubation at viral optimal temperature, the medium was removed and the cells were overlaid with plaquing medium (0.8% agarose in L-15 containing 2% FBS and 1% antibiotics). When the plaques appeared obviously, the cells were fixed in 10% formalin for 2 h, subsequently the agarose overlay was removed, and cells were stained with 10% crystal violet at room temperature. After washing the stained cells with distilled water, the plaque-forming unit (PFU) was calculated.

8. Neutralization assay

Prior to neutralization assay, serum samples were heated at 56°C for 30 min to inactivate complement. The inactivated serum (60 ㎕/well) was 2-fold serially diluted in L-15 containing 1% antibiotics and mixed with an equal volume of virus (1×10^3 PFU/60 ㎕/well) in U-shaped 96-well plates. The mixtures were incubated for 24 h at the viral optimal temperature then 100 ㎕ of each mixture was added to duplicate wells containing EPC cells monolayer. At 24 h and 48 h post inoculation, cell fluorescence was observed by fluorescent microscopy.

In the conventional neutralization assay using wild type HIRRV, serum samples were heated then 2-fold serially diluted. Sera were mixed with the virus (1×10^1 PFU/60 ㎕) and inoculated into fresh cells after incubation for 24 h at 15°C. CPE was observed at 3 days to 5 days post inoculation.

9. Statistical analysis

Viral growth and conventional neutralization data were analysed by Student's *t*-test. *P*-value of less than 0.05 was considered to be statistically significant.

Table 3. Summary of primers used in this study

3-1. For construction of N gene-eGFP gene-P gene cassette

Name	Sequence (5'-3')
SHRV_N_F	GGATCCCGAGGGAAACCACAG
SHRV_N_mid_R	CATGGTACCCCTCCTGAAGGTTCTCTTGTGTCT
N-eGFP_OL_F	**TTCAGGAGGGGTACC**ATGGTGAGCAAGGGCGAG
N-eGFP_OL_R	**AGTTCTGTTACGCGT**TTACTTGTACAGCTCGTCC ATGC
SHRV_P_F	TAAACGCGTAACAGAACTTCCCCCCTAACGAGTG
SHRV_P_mid_R	CTTAAGTTGTCTAATGTACTGTCTCAGTCTTTTG

(Bolded nucleotides indicate overlapped sequences)

3-2. For construction of pSHRV-Gvhsv-eGFP

Name	Sequence (5'-3')
SHRV_N_F	GGATCCCGAGGGAAACCACAG
SHRV_P_OC_R	CTTAAGTTGTCTAATGTACTGTCTCAGTCTTTTG
SHRV_P_OC_F	CATTAGACAACTTAAGATGAGCCATC
SHRV_L_mid_R	TAGGTGATGAGGACAATGGAGGTC
SHRV_L_mid_F	CCATTGTCCTCATCACCTAACCC
SHRV_N_R	TTTCCCTCGGGATCCTTTGAAAGC

3-3. For reverse-transcription PCR (RT-PCR) analysis

Name	Sequence (5'-3')
eGFP_696_F	CGGCATGGACGAGCTGTACAAGTAA
SHRV_P_R	GCGGCCGCTCACTTTGTGAGTTCAGCCTTCG
SHRV_M_mid_F	ACACCACCCAGATCAGGAAT
VHSV_G_mid_R	TATCTTCCCAGAGGCAGTGA
SHRV_G_SP_F	CGTCGACGATGACGCTCCCAAACATGAAAC
SHRV_NV_R	AACAACAACAATTGCCTACCAGGAGTCGTTGTCTTC
T_vector_2599_F	CCACTACGTGAACCATCACCC
SHRV_N_R	GCGGCCGCTTAGGCATACTTGCTGTAGTCCTGA

Ⅲ. Results

1. Western blot analysis

To verify whether the antibodies against VHSV bind to the G protein of rSHRV-Gvhsv, the virus was analysed by western blot using polyclonal rabbit anti-VHSV serum. In western blot analysis, wild-type VHSV was analysed together as a positive control.

We could observe that a band corresponding to the predicted protein molecular weight (MW) of the G protein appeared in rSHRV-Gvhsv infected cell lysate. In the lysate of infected cells with wild type VHSV, the bands of N, M and G protein were observed (Fig. 8). This result indicated that the neutralizing antibodies against VHSV could bind to the VHSV G protein of rSHRV-Gvhsv.

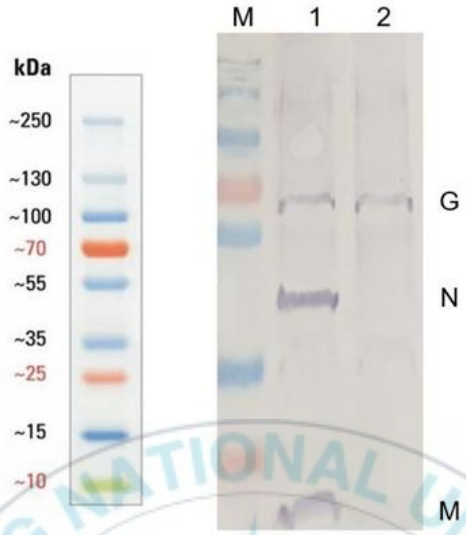


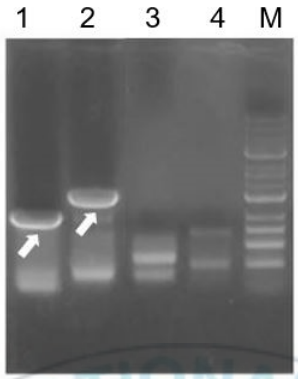
Fig 8. Result of western blot analysis. Protein samples were obtained from the cell pellets which were infected with wild-type VHSV (Lane 1) and rSHRV-Gvhsv (Lane 2). The marker is a PageRuler Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Scientific). G : glycoprotein, N : nucleoprotein, M : matrix protein.

2. Generation of rSHRV-Gvhsv expressing eGFP

In western blot analysis, we confirmed that the G protein of rSHRV-Gvhsv reacted to VHSV antisera. To use this chimeric virus for rapid neutralization assay, we generated recombinant rhabdovirus expressing reporter protein by insertion of eGFP gene into viral genome. For achieving high expression levels of eGFP in VHSV genome, we inserted the reporter gene between N gene and P gene. To produce recombinant virus, EPC cells expressing T7 RNA polymerase were transfected with a mixture of viral and helper plasmids. Through several passages on fresh cells, we could observe a virus-induced CPE and the eGFP expression in infected cells.

From the results of RT-PCR, sequencing and plaque assay, we confirmed the production of recombinant virus. In RT-PCR analysis, the bands of eGFP to P gene and M to VHSV G gene were successfully amplified from the viral stock (Fig. 9. A). To verify the sequence of rescued virus, the amplified bands were purified and analysed by nucleotide sequencing. In plaque assay, we could confirm a high level of viral titer (7×10^6 PFU/㎖) in cells inoculated with viral stock (Fig. 9. B).

(A)



(B)

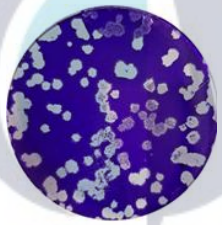
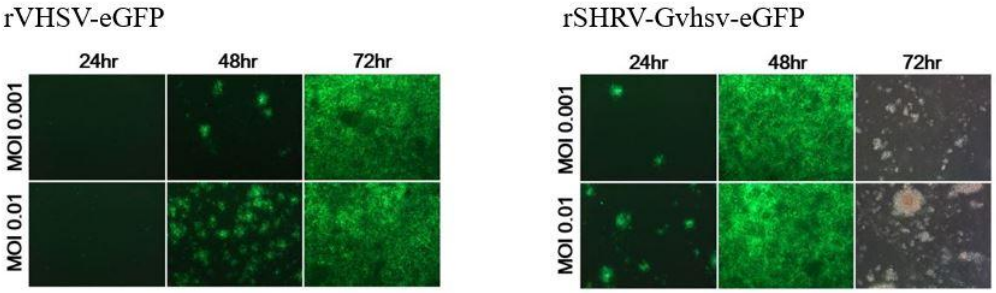


Fig 9. Confirmation of rescued rSHRV-Gvhsv-eGFP by RT-PCR and plaque assay. **(A)** The eGFP to P gene and M to VHSV G gene were successfully detected and the amplified DNA analysed by nucleotide sequencing. Lane 1 : eGFP to SHRV P gene (908 bp), Lane 2 : SHRV M gene to VHSV G gene (1.5 kb), Lane 3 : SHRV G gene (No band), Lane 4 : Plasmid backbone (No band). **(B)** Plaque formation in EPC cells infected with 10^5 diluted rSHRV-Gvhsv-eGFP.

3. Replication kinetics of recombinant viruses

To compare the replication ability of rSHRV-Gvhsv-eGFP with rVHSV expressing eGFP (rVHSV-eGFP), EPC cells were infected with rSHRV-Gvhsv-eGFP and rVHSV-eGFP at the multiplicity of infection (MOI) of 0.01 and 0.001. The cells were incubated at viral optimal temperature (rSHRV-Gvhsv-eGFP; 20°C, rVHSV-eGFP; 15°C), and supernatant was collected at 24 h, 48 h and 72 h post infection. Virus in collected supernatant was quantified in EPC cells by plaque assay. The results showed that the growth and final titer of rSHRV-Gvhsv-eGFP were significantly higher than those of rVHSV-eGFP in EPC cells (Fig. 10).

(A)



(B)

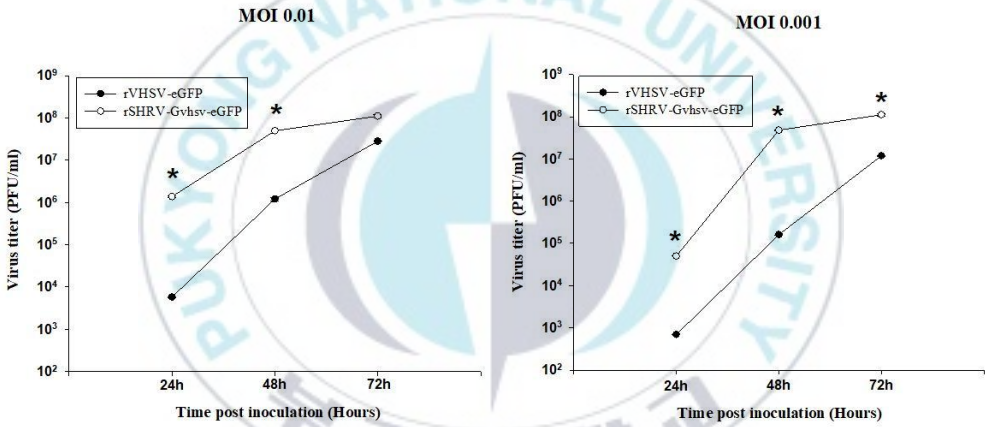


Fig 10. Replication kinetics of rVHSV-eGFP and rSHRV-Gvhsv-eGFP in EPC cells. Cells were infected at the multiplicity of infection (MOI) of 0.01 and 0.001. At 24 h, 48 h and 72 h post infection, the supernatant was collected and the virus titer analysed by plaque assay. **(A)** Observation of fluorescence of cells infected with rVHSV-eGFP and rSHRV-Gvhsv-eGFP. **(B)** Growth curves of rVHSV-eGFP and rSHRV-Gvhsv-eGFP. Asterisks represent significantly different at $P < 0.05$.

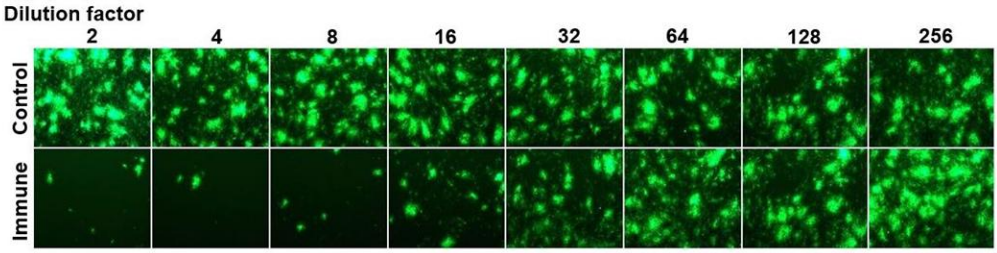
4. Neutralization assay

First of all, we performed the neutralization assay using polyclonal rabbit anti-VHSV sera to investigate whether the infectivity of rSHRV-Gvhsv-eGFP could be inhibited by the antibodies against VHSV. A naive serum from rabbit was used as a negative control. As expected, the neutralization activity was observed in immune serum-rSHRV-Gvhsv-eGFP mixture inoculated cells. In addition, we could confirm the results in 24 h post inoculation while the fluorescence was shown at 48 h in cells inoculated with rVHSV-eGFP. It enables us to perform rapid neutralization assay using chimeric rhabdovirus to quantify neutralizing antibodies against VHSV (Fig. 11-1).

Furthermore, to confirm the possibility of the chimeric rhabdovirus expressing eGFP for the practical use, neutralization assay based on rSHRV-Gvhsv-eGFP was performed using sera from olive flounders immunized with VHSV. The results showed that rSHRV-Gvhsv-eGFP could be successfully neutralized by the antibodies produced by immunization, indicating that rSHRV-Gvhsv-eGFP could be used for detection of neutralization activity against VHSV (Fig. 11-2).

Added to this, to confirm whether rSHRV-Gvhsv-eGFP can be used for investigation of recent exposure to VHSV, neutralization assay was carried out using sera extracted from wild olive flounders. As a result, the fish that showed neutralization activity against rVHSV-eGFP indicated neutralization activity against rSHRV-Gvhsv-eGFP (Fig. 11-3). Using this serum, we performed conventional neutralization assay against HIRRV to verify the specificity of rSHRV-Gvhsv-eGFP to antisera of VHSV. As shown in Fig. 11-4, neutralization activity against HIRRV was not observed, therefore, neutralization assay using rSHRV-Gvhsv-eGFP seems to be specific to neutralizing antibodies against VHSV.

(A)



(B)

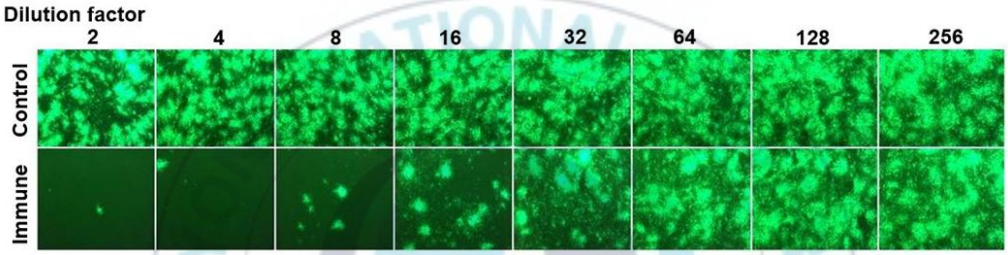
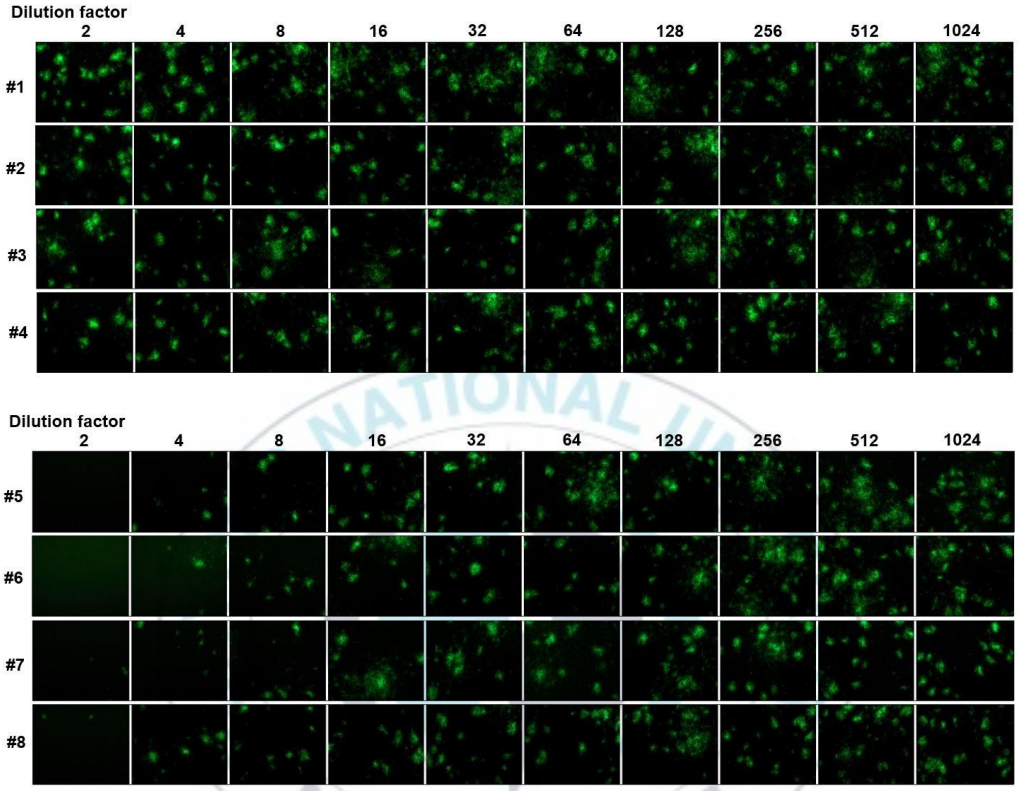


Fig 11-1. Results of neutralization assay using rabbit sera. Control serum was obtained from naive rabbits and immune serum was extracted from immunized rabbits by infection of formalin-inactivated VHSV and additional boosting. Serum samples were 2-fold serially diluted then mixed with the recombinant virus and incubated at viral optimal temperature. (A) The fluorescence of cells inoculated with rabbit sera and rSHRV-Gvhsv-eGFP. Cell fluorescence appeared in 24 h post inoculation. (B) The fluorescence of cells inoculated with rabbit sera and rVHSV-eGFP. Fluorescence could be observed at 48 h post inoculation.

(A)


Dilution factor
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#8

(B)

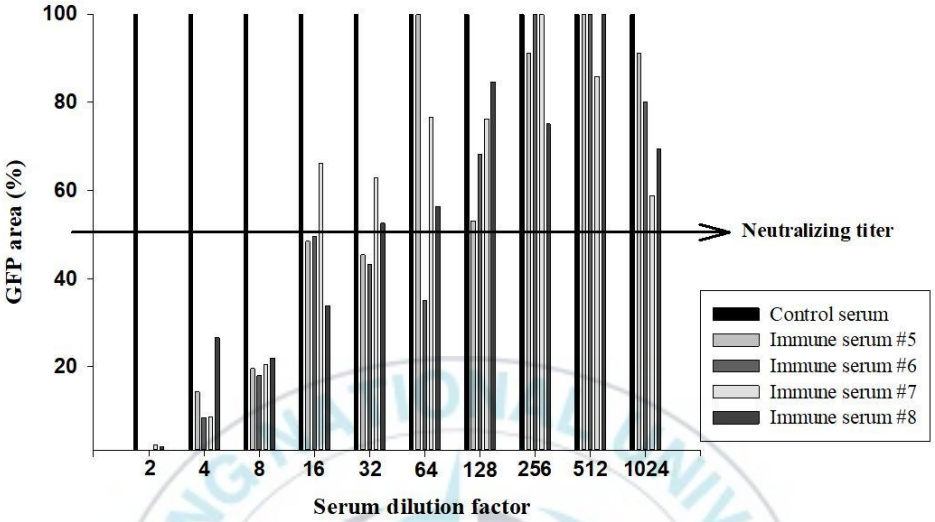
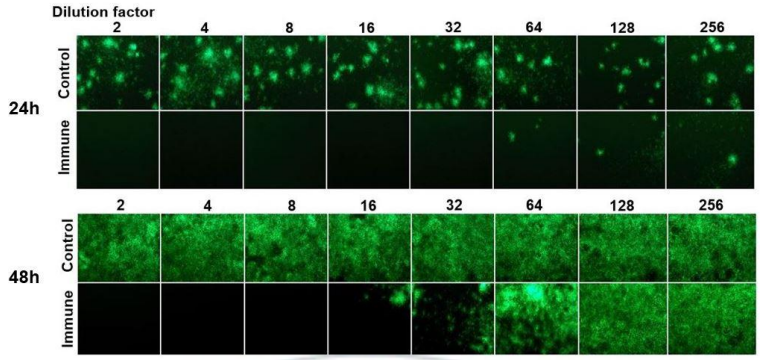


Fig 11-2. Results of neutralization assay using sera from olive flounders (*Paralichthys olivaceus*) immunized with wild-type VHSV. Serum samples were 2-fold serially diluted and mixed with rSHRV-Gvhsv-eGFP. The mixtures were incubated at 20℃ for 24 h then inoculated into EPC cells. (#1, 2, 3, 4 : Cells infected with control sera. #5, 6, 7, 8 : Cells infected with immune sera) **(A)** Cell fluorescence at 24 h post inoculation **(B)** Quantitation of GFP area (%) from the cells which appeared fluorescence. Analysis of GFP area was performed using Image-Pro Plus and the fluorescence of cells inoculated with #1 control serum was considered as 100% quantity of fluorescence. The neutralizing titer was determined at the point of which the fluorescent area was lower than 50% of cells inoculated with control serum.

(A)



(B)

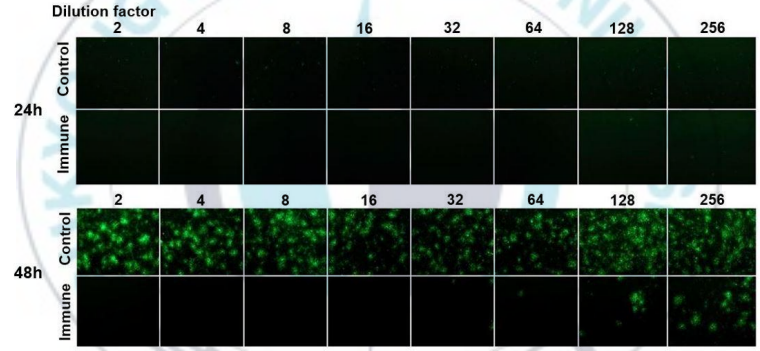


Fig 11-3. Results of neutralization assay for investigation of the exposure of VHSV. Serum samples were extracted from wild olive flounders (*Paralichthys olivaceus*) and used for neutralization assay using rSHRV-Gvhsv-eGFP and rVHSV-eGFP. **(A)** Result of neutralization assay using rSHRV-Gvhsv-eGFP. The fluorescence appeared at 24 h post inoculation. **(B)** Result of neutralization assay using rVHSV-eGFP. The fluorescence appeared at 48 h post inoculation.

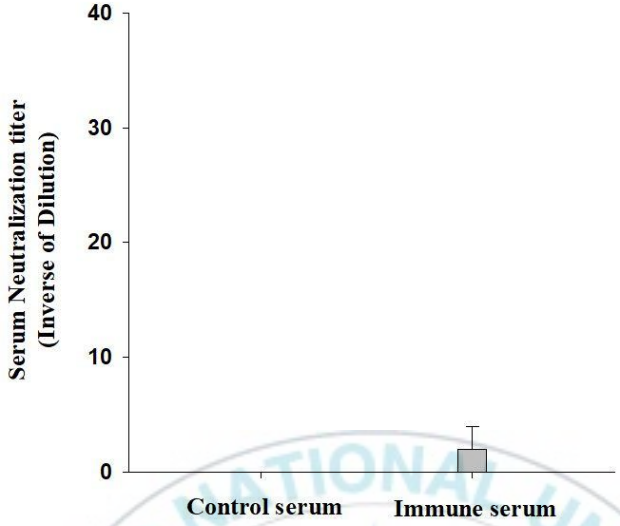


Fig 11-4. Serum neutralization titer against HIRRV. Conventional neutralization assay was performed using serum which showed neutralization activity against both rSHRV-Gvhsv-eGFP and rVHSV-eGFP. Significant difference from control group was analysed using student's *t*-test.

Ⅳ. Discussion

To date, various types of vaccines against VHSV have been reported, such as whole-virus inactivated vaccines, attenuated vaccines, subunit vaccines, recombinant G protein-based vaccines, genetically engineered viruses and DNA vaccines (Gomez-Casado et al., 2011; Kim and Kim, 2019). To determine successful vaccination, the presence of antibodies in sera to bind and neutralize VHSV should be verified. Also, not only investigation of previous exposure to VHSV but also serological diagnosis is performed by detecting NAbs in serum samples.

Among several methods for detection and quantitation of NAbs, such as virus neutralization assay, plaque reduction neutralization test (PRNT) and enzyme-linked immunosorbent assay (ELISA) (Wilson et al., 2014), the virus neutralization assay has been used commonly to detect NAbs, but it has several disadvantages that are difficult to be resolved. In addition, in the case of neutralization assay of VHSV, a lot of time is required due to slow replication of VHSV.

In the present study, we aimed to use rSHRV-Gvhsv which replicates faster than VHSV as a challenge virus to develop a rapid neutralization assay of VHSV. In novirhabdoviruses, it has been reported that the neutralization activity against chimeric recombinant viruses expressing heterologous glycoprotein is dependent on the corresponding G protein of the virus. Romero et al. (2005) found that rIHNV-Gvhsv could produce specific VHSV antibodies but not IHNV antibodies, and Kim and Kim (2012) confirmed that the fish immunized with rVHSV-Ghirrv-△NV-eGFP showed high neutralizing titer against HIRRV. In our western blot analysis, it was verified that the

antibodies against VHSV bound to the G protein of rSHRV expressing VHSV G protein, suggesting that the chimeric virus expressing VHSV G protein could be used for quantitation of NAbs against VHSV.

Recently, neutralization assay based on recombinant virus expressing reporter protein has been utilized widely as an alternative to present neutralization assays in mammalian rhabdoviruses including rabies virus (RABV) and vesicular stomatitis virus (VSV). The assay provides rapid and high-throughput results and indicates corresponding neutralizing antibody titer to the conventional neutralization test (Burgado et al., 2018; Tang et al., 2015; Qin et al., 2019). Likewise, to make the neutralization assay for VHSV more simple and high-throughput, we constructed rSHRV-Gvhsv expressing eGFP by inserting the eGFP gene between N gene and P gene for a high expression of reporter protein and assessed its possibility as a challenge virus for the neutralization assay of VHSV. In the neutralization assay using rSHRV-Gvhsv-eGFP, the infection of chimeric rhabdovirus expressing eGFP was successfully blocked by the antibodies against VHSV and the neutralization activity could be detected easily based on the fluorescence expression by confocal microscopy. Furthermore, the specificity of the chimeric virus to VHSV antibodies was demonstrated in that sera which showed neutralization activities against rSHRV-Gvhsv-eGFP and rVHSV-eGFP did not show any neutralization activities against HIRRV.

In conclusion, we developed rapid neutralization assay of VHSV based on chimeric rhabdovirus expressing eGFP (rSHRV-Gvhsv-eGFP). When rSHRV-Gvhsv-eGFP was used for neutralization assay of VHSV, we could obtain the results in 24 h post inoculation while the conventional methods that use wild type VHSV as a challenge virus need at least 5 days. Further, the assay did not require any additional steps in determination of neutralizing antibody titer since the fluorescence in cells infected with the chimeric virus

expressing eGFP could be visualized directly under a fluorescent microscope. Moreover, as the assay is considered to be specific to antibodies against VHSV, the assay can be used not only for evaluation of vaccine candidates but also for screening of viral exposure history. However, in our assay, one disadvantage for rSHRV-Gvhsv-eGFP is that the virus might be not safe as rSHRV-Gvhsv showed high virulence in zebrafish in previous study. Hence, the effort to make the virus avirulent is necessary for its practical use in neutralization assay.

Generation and characterization of chimeric rhabdoviruses expressing heterologous glycoproteins and Development of rapid neutralization assay using chimeric rhabdovirus

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Abstract

Rhabdoviruses are enveloped, non-segmented, negative single-stranded RNA viruses, and some of them such as infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), snakehead rhabdovirus (SHRV), hirame rhabdovirus (HIRRV), and spring viremia of carp virus (SVCV) were known as fish rhabdovirus. With regard to the outbreak of fish rhabdoviral diseases, the water temperature plays a significant role; the occurrences of IHNV, VHSV and HIRRV are below 20°C, whereas SHRV occurs at warm water temperatures between 28°C to 31°C. SVCV usually occurs in spring at water temperature below 20°C, while it can replicate in cell cultures up to 31°C.

To date, several studies have been performed to investigate the determinants for temperature sensitivity and virulence in fish rhabdoviruses. However, the determinants were not revealed clearly and the suggestions were different depending on the virus species and strains. Therefore, in the present study, among the viral proteins consisting of rhabdovirus, the glycoprotein (G) which is the major surface antigen exposed at the viral envelope and is responsible

for multiple-critical roles in viral infection and replication, was studied to examine its function in determination of viral replication temperature and *in vivo* virulence. To accomplish this task, several chimeric rhabdoviruses expressing heterologous glycoproteins (rSHRV-Gvhsv, rSHRV-Gsvcv, rVHSV-Gshrv and rVHSV-Gsvcv) were generated using reverse genetics, their characteristics in EPC (*Epithelioma papulosum cyprini*) cells were evaluated at different temperatures and their virulence in zebrafish was determined.

In EPC cells, the viral replication and plaque formation of rSHRV-Gvhsv were restricted to low temperatures, and rVHSV-Gshrv and rVHSV-Gsvcv showed high replication ability at 20°C, which is higher than the optimal temperature of VHSV, but the range of replication temperatures was not changed. Consequently, the results suggest that the G protein of VHSV would participate in the determination of low-temperature growth. However, considering the characteristics of chimeric VHSVs, the G protein alone is not enough to determine the temperature sensitivity, suggesting that not only G protein but also other proteins or factors might be involved in the determination of low-temperature sensitivity.

In order to investigate other proteins associated with the replication temperature of VHSV, we constructed SHRV minigenome and co-transfected into EPC cells with homologous and heterologous combinations of N, P and L helper plasmids. As a result, we found that N protein and P protein are not likely to be responsible for the determination of viral temperature, while L protein is highly related to the low-temperature replication in VHSV.

In zebrafish challenge test, VHSV lost its infectivity when the glycoprotein was exchanged with the SHRV or SVCV while SHRV gained high virulence at low-temperatures when substituted its glycoprotein with VHSV G protein. Meanwhile, although SVCV caused mortalities in all fish, no mortality was

shown in rSHRV-Gsvcv infected fish. These results imply that the VHSV G protein is deeply involved in viral infectivity and virulence and the SHRV and SVCV G protein might not be directly related to their virulence, suggesting that some other viral proteins or factors other than the G protein could be responsible for determination of the virulence.

Furthermore, since the glycoprotein is the major surface viral protein recognized by neutralizing antibodies (NAbs) and the NAbs are induced exclusively against the G protein, we aimed to use the chimeric rhabdovirus expressing heterologous glycoprotein for the neutralization assay. In the present study, rSHRV expressing VHSV G protein was used for rapid neutralization assay for VHSV as the chimeric virus has higher replication ability compared to VHSV. In western blot analysis, we confirmed that the NAbs against VHSV could bind to the VHSV G protein of chimeric virus, indicating the availability for the challenge virus in neutralization assay for VHSV.

Moreover, to make the neutralization assay for VHSV more simple and high-throughput, we constructed rSHRV-Gvhsv expressing enhanced green fluorescent protein (eGFP) by inserting the eGFP gene between N gene and P gene, and assessed its possibility as a challenge virus for VHSV neutralization assay. In the neutralization assay using rSHRV-Gvhsv-eGFP, the infection of chimeric virus was successfully blocked by the antibodies against VHSV and the neutralization activity could be detected easily based on the fluorescence expression by confocal microscopy. Furthermore, the specificity of the chimeric virus to VHSV antibodies was demonstrated in that sera which showed neutralization activities against rSHRV-Gvhsv-eGFP and rVHSV-eGFP did not show any neutralization activities against HIRRV. Taken together, the rapid neutralization assay developed in this study could rapidly quantify the levels of neutralizing antibodies of VHSV, furthermore, the assay could show the results in 24 h post inoculation while the conventional methods that use wild-type

VHSV as a challenge virus need at least 5 days. Further, the assay did not require any additional steps in determination of neutralizing antibody titer since the fluorescence in cells infected with the chimeric virus expressing eGFP could be visualized directly under a fluorescent microscope.

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