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Generation of VHSV replicon particles carrying transmembrane and C-terminal cytoplasmic region-deleted G gene (rVHSV-GΔTM) and comparison of vaccine efficacy with G gene-deleted VHSV (rVHSV-ΔG)

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ABSTRACT

Vaccines based on viral replicon particles would be advantageous to induce immune responses compared to inactivated viruses in that they can infect host cells (only once) and can produce viral proteins in the infected cells like live viruses. Furthermore, as viral replicon particles are replication-defective, they are safer than live attenuated viruses. Previously, we had rescued viral hemorrhagic septicemia virus (VHSV) replicon particles lacking full ORF of G gene (rVHSV-ΔG). In the present study, to enhance the immunogenicity of VHSV replicon particles, we newly generated another form of VHSV replicon particles that can produce the transmembrane and C-terminal cytoplasmic region-deleted G protein in host cells (rVHSV-GΔTM), and compared the protective efficacy of rVHSV-GΔTM with that of rVHSV-ΔG through immunization of olive flounder (*Paralichthys olivaceus*). In addition, we evaluated the safety of rVHSV-GΔTM by the analysis of effects on wild-type VHSV replication. In the vaccine experiment, olive flounder immunized with rVHSV-GΔTM showed significantly higher titers of serum neutralization activity than fish immunized with rVHSV-ΔG suggesting that the G protein that is not only spiked on the viral envelop but also secreted extracellularly can contribute to the enhancement of adaptive humoral immunity. Moreover, fish immunized with rVHSV-GΔTM showed higher survival rates than fish immunized with rVHSV-ΔG, suggesting that the amount of G protein provided to hosts is an important factor for the enhancement of vaccine efficacy against VHSV disease. In a safety aspect, rVHSV-GΔTM could not replicate in infected cells, and significantly inhibited the replication of wild-type VHSV when co-infected, suggesting that rVHSV-GΔTM would not worsen disease progression caused by wild-type VHSV infection.

1. Introduction

Viral hemorrhagic septicemia virus (VHSV) in the genus *Novirhabdovirus* of the family Rhabdoviridae has afflicted freshwater and marine fishes world-wide [1–3]. In Korea, the culture farms of olive flounder (*Paralichthys olivaceus*) have been damaged by frequent outbreaks of VHSV disease during low temperature periods [4]. The genome of VHSV consists of 6 genes encoding nucleoprotein N, phosphoprotein P, matrix protein M, glycoprotein G, nonvirion protein NV, and RNA-dependent RNA polymerase L [5]. The N, P, and L proteins cooperate to replicate the whole genome and to transcript viral genes [6]. The G protein spiked on the viral envelope plays a critical role in the infection of VHSV through binding to cell membrane receptor and fusion to endosomal membrane at low pH to release viral nucleocapsids into cytoplasm [7]. As the G protein is the only antigen that can induce neutralization antibodies in hosts, G protein has been the main target for the development of prophylactic vaccines against rhabdoviruses. Various types of vaccine have been tried to prevent VHSV disease in cultured fishes, such as whole-virus inactivated vaccines, attenuated vaccines, recombinant G protein-based vaccines, and DNA vaccines [2,8]. However, there are still no available VHSV vaccines approved for the commercial use in olive flounder farms.

Previously, using the reverse genetic technology, we had rescued an attenuated VHSV that had the enhanced green fluorescent protein (eGFP) gene ORF instead of the NV gene ORF in the genome (rVHSV-ΔNV-EGFP), and had showed a high vaccine potential of the recombinant VHSV [9,10]. However, due to the replicability of rVHSV-ΔNV-EGFP in host cells, we could not completely rule out the possibility that immunologically weakened fishes can suffer from the disease caused by rVHSV-ΔNV-EGFP. To solve the safety problem related to viral replication in host cells, we had produced whole G gene ORF-deleted VHSV replicon particles (rVHSV-ΔG) that had no ability to

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produce infective viral particles. The vaccine potential and safety of rVHSV-ΔG were demonstrated through immunization of olive flounder and in vitro & in vivo co-infection with wild-type VHSV, respectively [11,12]. However, as all the G protein of rVHSV-ΔG is just *trans*-supplied from G protein-expressing cells and rVHSV-ΔG has no ability to express G protein in infected cells, rVHSV-ΔG can provide only a limited amount of G protein to hosts. Therefore, in the present study, we newly generated another form of replicon particles of VHSV that can produce the transmembrane and C-terminal cytoplasmic region-deleted G protein in host cells (rVHSV-GΔTM), and compared the protective efficacy of rVHSV-GΔTM with that of rVHSV-ΔG through immunization of olive flounder. Furthermore, we evaluated the safety of rVHSV-GΔTM by the analysis of effects on wild-type VHSV replication. The results showed that olive flounder immunized with rVHSV-GΔTM induced higher survival rates than fish immunized with rVHSV-ΔG, and the replication of wild-type VHSV was significantly inhibited by co-infection with rVHSV-GΔTM.

2. Materials and methods

2.1. Cells and viruses

Epithelioma papulosum cyprini (EPC) cells and G protein-expressing EPC cells (used for the culture of rVHSV-ΔG [12]) were maintained in Leibovitz medium (L-15, Sigma) supplemented with 10% fetal bovine serum (FBS, Welgene) and penicillin-streptomycin (Gibco). The wild-type VHSV KJ2008 was propagated in monolayer of EPC and replication-defective recombinant VHSVs (rVHSV-ΔG and rVHSV-GΔTM) were grown in monolayer of G protein-expressing EPC cells at 15°C in the presence of 2% FBS.

2.2. Generation of rVHSV-GΔTM

Single-cycle rVHSV lacking transmembrane and C-terminal cytoplasmic region of G gene in the genome (rVHSV-GΔTM) was recovered through following procedures. Briefly, the fragment of transmembrane and C-terminal cytoplasmic region-deleted G gene (GΔTM) was PCR amplified using a previously constructed vector, pVHSV-wild [9], as a template and a specific primer pair in Table 1. The amplified PCR product was cloned into the pGEM T-easy vector (Promega) and sequenced. The T vector was digested with *Age*I and *Sac*II, and the digested fragment encoding the transmembrane and C-terminal cytoplasmic region-deleted G gene was ligated to the pVHSV-wild vector that was predigested with the same enzymes, resulting in pVHSV-GΔTM. EPC cells expressing T7 RNA polymerase [9] were grown to about 80% confluence and transfected with a mixture of pVHSV-GΔTM (2 μg) and helper plasmids - pCMV-N (500 ng), pCMV-P (300 ng) and pCMV-L (200 ng) - using Fugene HD transfection reagent (Promega) according to manufacturer's instructions. After incubation for 12 h at 28°C, the transfected cells were shifted to 15°C. When extensive cytopathic effect (CPE) was observed, the supernatant was harvested and passaged at least 3 times in G protein-expressing EPC cells.

2.3. Semi-quantitative RT-PCR

The expression of transmembrane and C-terminal cytoplasmic region-deleted G gene in EPC cells infected with rVHSV-GΔTM was analyzed using semi-quantitative RT-PCR. EPC cells seeded in 35-mm dish (1.5×10^6 cells/dish) were infected with each rVHSV-ΔG or rVHSV-GΔTM at a MOI of 0.1, and incubated at 15 °C. At 48 h post-infection, total RNA was extracted using Hybrid-R Kit (GeneAll, Korea) and complementary DNA (cDNA) was synthesized using M-MLV RT Master Mix (ELPIS, Korea) according to the manufacturer's instruction. The semi-quantitative RT-PCR was carried out using the PCR primer sets in Table 1. Thermal cycling condition was 1 cycle of 3 min at 95°C (initial denaturation), followed by 30 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, with a final extension step of 7 min at 72°C. PCR samples were electrophoresed on the 1% agarose gel.

2.4. Western blot analysis to confirm the amount of extracellular G protein by rVHSV-GΔTM infection

EPC cells (1.5×10^6 cells/35-mm dish) were infected with wild-type VHSV, rVHSV-ΔG, rVHSV-GΔTM at a MOI of 0.1, respectively, and incubated at 15 °C. After 24 h, the cells infected with each virus were rinsed 3 times with phosphate-buffered saline (PBS), and supplemented with fresh L-15 medium. At 5 days post-inoculation, each virus supernatant was harvested, and viral proteins were boiled at 95°C for 5 min with PAGESTA REDUCING 5× SDS sample buffer (GeneAll), and loaded on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane. The membrane was blocked in blocking solution (3% bovine serum albumin in TBS; 150 mM NaCl, 10 mM Tris-HCl,

Table 1
Summary of primers used in this study.

Name of primer			Sequence (5′ to 3′)
For construction of pVHSV-GΔTM			
GΔTM	F-*Age*I		ACCGGTATGGAATGGAATACTTTTTTCTTG
	R-*Sac*II		CCGCGGTCAACTCCAATTGAATGACCAATCCGAAGG
For verification of production of rVHSV-GΔTM by RT-PCR			
VM		F	CCAGGTCGATAAGATCTGCATG
VG		R1	GCCTTGACCACCCTGTGATCATGTGTC
		R2	TCAGACCATCTGGCTTCTGGAGAACTG
For comparison of G gene expression by semi-quantitative RT-PCR			
VG		F	CCTCCAACTGCAGAAAAAACTCC
		R	GCCTTGACCACCCTGTGATCATGTGTC
VP		F	GGGTCCTCCAAACAGAAGCCAAGCCCCAAG
		R	CTACTCCAACTTGTCCAACTCCG
BA (β-actin)		F	AAGGAGAAGCTCTGCTATGTGGCT
		R	AAGGTGGTCTCATGGATACCGCAA
For estimation of the expression level of the type I interferon by real-time RT-PCR			
ISG15		F	TGATGCAAATGAGACCGTAGAT
		R	CAGTTGTCTGCCGTTGTAAATC
Mx1		F	TGGAGGAACCTGCCTTAAATAC
		R	GTCTTTGCTGTTGTCAGAAGATTAG
For confirmation of rVHSV-GΔTM genome in EPC cells by RT-PCR			
VG		F	GCAACAGACGACTTCTCCTATC
VNV		R	GGTCCTTAGATCCTCTGAGACT

Underlined characters represent restriction enzyme sites.

pH 7.5) for 2 h at room temperature (RT), and rinsed with TTBS (0.05% Tween 20 in TBS, pH 7.5), then, incubated with diluted rabbit anti-VHSV G antiserum (1:500) for 2 h at RT. The rabbit anti-VHSV G antiserum was obtained by the immunization of rabbits with recombinant G protein. The membrane was rinsed 3 times with TTBS and incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (1:2000, Abcam) for 2 h at RT. After rinsing 3 times with TTBS, the specific antigen-bound antibody was visualized with KPL 5-bromo-4-chloro-3-indoly phosphate and nitroblue tetrazolium (BCIP/NBT) phosphatase substrate (SeraCare).

2.5. RT-PCR analysis to confirm the presence of intra- and extracellular rVHSV-GΔTM

EPC cells (1.5×10^6 cells/35-mm dish) were infected with rVHSV-GΔTM at a MOI of 0.1 and incubated at 15°C (1st passage). After 24 h, the cells were washed 3 times with PBS, and supplemented with fresh L-15 medium. At 3 and 7 days post-inoculation, supernatant and cell pellets were harvested. Total RNA was extracted from 1 ml of the supernatant according to the Trizol RNA isolation protocol, and from the cell pellets using Hybrid-R Kit (GeneAll). Complementary DNA (cDNA) was synthesized using M-MLV RT Master Mix (ELPIS) according to the manufacturer's instruction. PCR was carried out using the PCR primer sets in Table 1. Thermal cycling condition was 1 cycle of 3 min at 95°C (initial denaturation), followed by 30 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, with a final extension step of 7 min at 72°C. PCR samples were electrophoresed on 1% agarose gel.

To confirm whether the above supernatant obtained at 7 days post-infection contained infective rVHSV-GΔTM, EPC cells were exposed to the supernatant and incubated at 15°C. At 3 and 7 days post-infection, supernatant and cells were harvested, and performed RT-PCR as described above.

2.6. Effect of rVHSV-GΔTM on wild-type VHSV replication

EPC cells (1.5×10^6 cells/35-mm dish) were infected with wild-type VHSV alone at a MOI of 0.00001 or infected with wild-type VHSV (MOI 0.00001) plus rVHSV-GΔTM (MOI 0.1, 0.01, and 0.001), and incubated at 15°C. To know the effect of rVHSV-GΔTM on wild-type VHSV replication, each supernatant was harvested at 1, 3, 5, and 7 days post-infection, and plaque assay was performed. Briefly, EPC cell monolayers (1.5×10^6 cells/35-mm dish) were inoculated with serially diluted each supernatant, and incubated at 15°C for 1 h. And, then, the inoculum was removed and cells were overlaid with plaquing medium (0.8% agarose in L-15 containing antibiotics). After 7 days, the cells were fixed by 10% formalin and stained with 5% crystal violet for 30 min at room temperature. After rinsing of the cells with distilled water, the plaque-forming units (PFU) were counted.

2.7. Immunization and challenge

Olive flounder fingerlings (average body weight 9.2 g) were obtained from a local fish farm in Korea, and free from pathogens including VHSV was confirmed by the examination of randomly sampled 10 fish. After 2 weeks of acclimation, ninety fingerlings were randomly divided into 3 groups (15 fish/group) with 2 replicates, and were immunized with rVHSV-ΔG (1×10^4 PFU/fish), rVHSV-GΔTM (1×10^4 PFU/fish), or L-15 alone (control group) by intramuscular (i.m.) injection. Fish were kept at 18–20 °C. At 3 weeks post-immunization, 3 fish in each group were bled to obtain serum, and the other fish were gradually adapted to 13 °C over a week, and then intramuscularly challenged with the wild-type VHSV at 1×10^4 PFU/fish. Mortalities were recorded daily for 21 days post-infection.

2.8. Serum neutralization activity

The sera were heat-inactivated at 56°C for 30 min to inactivate complement before use in the test. The serially diluted sera were mixed with a fresh control serum obtained from VHSV-free healthy olive flounder, and incubated with wild-type VHSV in U-shaped 96-well at 15°C for overnight. And, then, 100 μl of each mixture was added to EPC cells monolayer, and observed CPE every day. The titer of each serum was the last dilution at which CPE was not observed.

2.9. Statistics

Statistical significance was analyzed using SPSS for Windows (Chicago, IL, USA). Data were analyzed by using one-way ANOVA followed by Tukey HSD post-hoc test. Kaplan-Meier method was used to the data on cumulative mortality, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Rescue of rVHSV-GΔTM

The pVHSV-GΔTM vector was constructed by the replacing of full G gene with the transmembrane and C-terminal cytoplasmic region-deleted G gene (GΔTM) (Fig. 1A), and replication-defective rVHSV-GΔTM particles were successfully rescued using full G gene-expressing EPC cells. The deletion of transmembrane and C-terminal cytoplasmic region of G gene in the genome of rVHSV-GΔTM was verified by RT-PCR, in which the band across M gene and the C-terminal cytoplasmic region of G gene was not amplified (Fig. 1B). Furthermore, EPC cells infected with rVHSV-ΔG showed no transcript of G gene, while cells infected with rVHSV-GΔTM showed a high amount of G gene transcript (Fig. 1C).

3.2. Western blot analysis to confirm the amount of extracellular G protein by rVHSV-GΔTM infection

In the supernatant of EPC cells infected with wild-type VHSV as well as rVHSV-GΔTM, the band corresponding to G protein was detected by Western blot (Fig. 2). However, no band was detected from the supernatant isolated from rVHSV-ΔG infected cells (Fig. 2).

3.3. RT-PCR analysis to confirm the presence of intra- and extracellular rVHSV-GΔTM

When EPC cells infected with rVHSV-GΔTM, the region ranging from G gene to NV gene was amplified from both supernatant and cell pellets (Fig. 3A). However, when EPC cells exposed to the supernatant obtained from the 1st passage of rVHSV-GΔTM, the amplification was detected only from the supernatant (Fig. 3B).

3.4. Effect of rVHSV-GΔTM on wild-type VHSV replication

As rVHSV-GΔTM could not form plaques without full G protein *trans*-supply, the effect of rVHSV-GΔTM on wild-type VHSV replication was determined by plaque numbers of wild-type VHSV. Although wild-type VHSV plaque numbers were not much influenced by the coinfection with rVHSV-GΔTM at MOI 0.001, the titers were significantly reduced when coinfected with rVHSV-GΔTM at MOI 0.1 or 0.01, and the decrease was proportional to the titers of rVHSV-GΔTM (Fig. 4A). The progression of cytopathic effect (CPE) was also clearly inhibited by coinfection with rVHSV-GΔTM at MOI 0.1 or 0.01 (Fig. 4B).

3.5. Protective efficacy and serum neutralization activity

In both replicates, the survival rates of olive flounder fingerlings

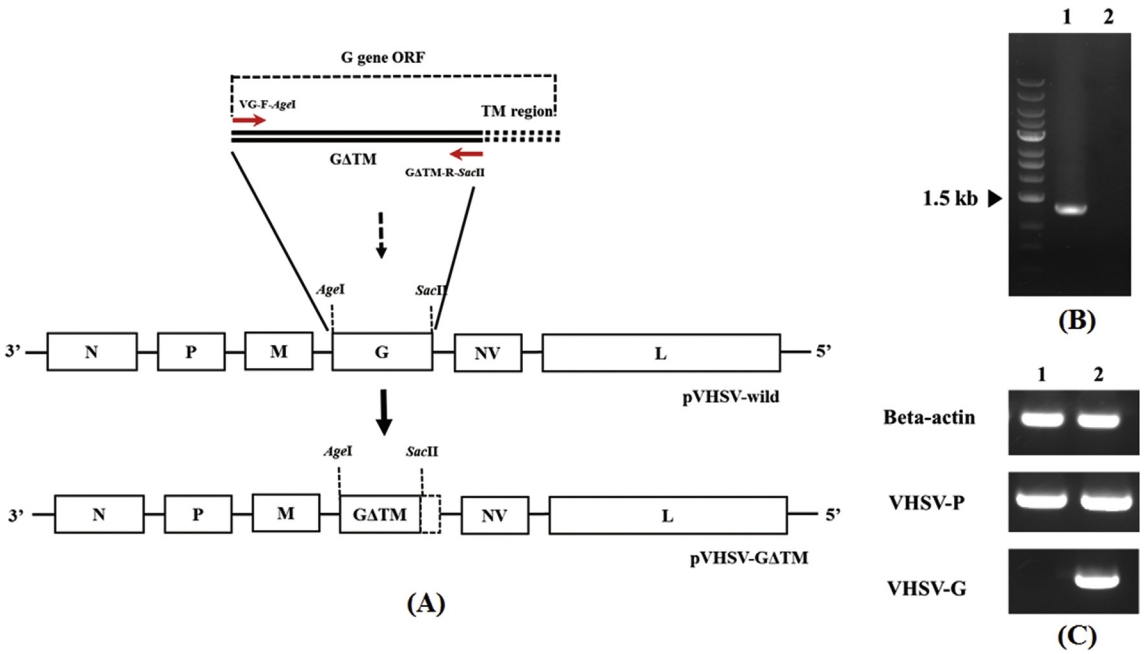

Fig. 1. (A) The schematic representation of a vector for the generation of VHSV replicon particles carrying transmembrane region-deleted G gene (rVHSV-GΔTM). (B) Verification of the transmembrane region deleted-G gene in the genome of rVHSV-GΔTM by RT-PCR. The band of lane 1 (approximately 1.3 kb) was amplified from M gene to the front of the transmembrane region of G gene. The region ranging from M gene to the end of G gene was not amplified (lane 2) due to the lacking of transmembrane region in the genome of rVHSV-GΔTM. (C) The G gene expression in EPC cells infected with rVHSV-GΔTM (lane 2) was verified by RT-PCR. Cells infected with rVHSV-ΔG did not show any transcription of G gene (lane 1). The expression of P gene in cells infected with rVHSV-ΔG was not different from that in cells infected with rVHSV-GΔTM.

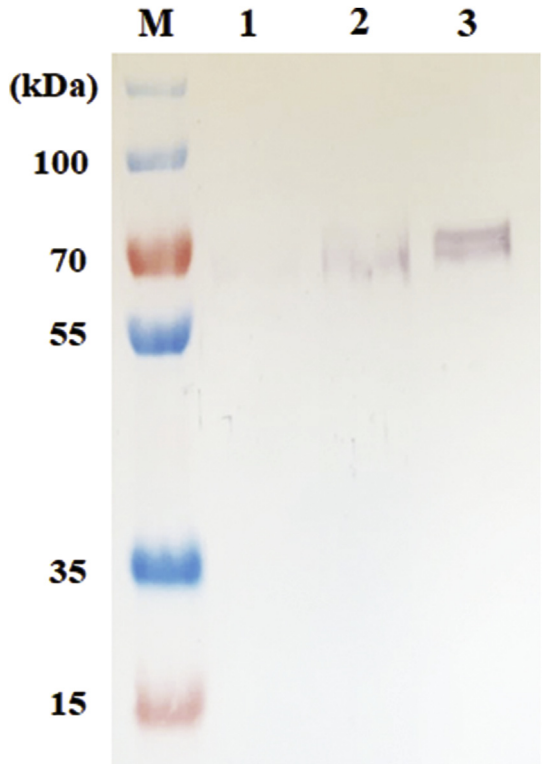

Fig. 2. The expression of G protein was analyzed by Western blot from the supernatant of EPC cells that were infected with rVHSV-ΔG (Lane 1), rVHSV-GΔTM (Lane 2), and wild-type VHSV (Lane 3). M, PageRuler prestained protein ladder (Thermo).

immunized with rVHSV-GΔTM or rVHSV-ΔG were significantly higher than those of control group fish that were injected with L-15 alone. Particularly, the group of fish immunized with rVHSV-GΔTM showed higher survival rates than the group of fish immunized with rVHSV-ΔG (Fig. 5). In addition, the serum neutralization titer of fish immunized with rVHSV-GΔTM was significantly higher than that of rVHSV-ΔG (Fig. 6).

4. Discussion

Despite inability to produce propagating viruses, viral replicon particles would be advantageous to induce immune responses compared to inactivated viruses in that they can infect host cells (only once though) and can produce viral proteins in the infected cells like live viruses. However, considering the critical role of rhabdoviral glycoprotein in the induction of protective immune responses, G gene-deleted VHSV replicon particles (rVHSV-ΔG) might not be the optimal form to elicit immune responses efficiently because they cannot express G protein in infected cells. As G protein is spiked on the viral envelop using its transmembrane region, the removal of the transmembrane region from G protein would lead to the secretion of G protein, which would make it impossible to produce replicative viral particles. In the present study, we successfully rescued G protein's transmembrane and C-terminal cytoplasmic region-removed recombinant VHSV (rVHSV-GΔTM) that could not replicate in cells without the *trans*-supply of full G proteins. The inability of rVHSV-GΔTM to infect cells without the supply of full G protein was demonstrated not only by the defect in plaque formation but also by the results that showing no amplification of a region of viral genome in the cells exposed to the supernatant collected from cells infected with rVHSV-GΔTM. In this study, the secretion of the truncated G protein was confirmed from supernatant by Western blot. Therefore, this single-cycle rVHSV-GΔTM can provide not only envelope-spiked G protein but also a secreted form of G protein to vaccinated fish, and we anticipated that rVHSV-GΔTM would be better than rVHSV-ΔG in inducing protective immunity.

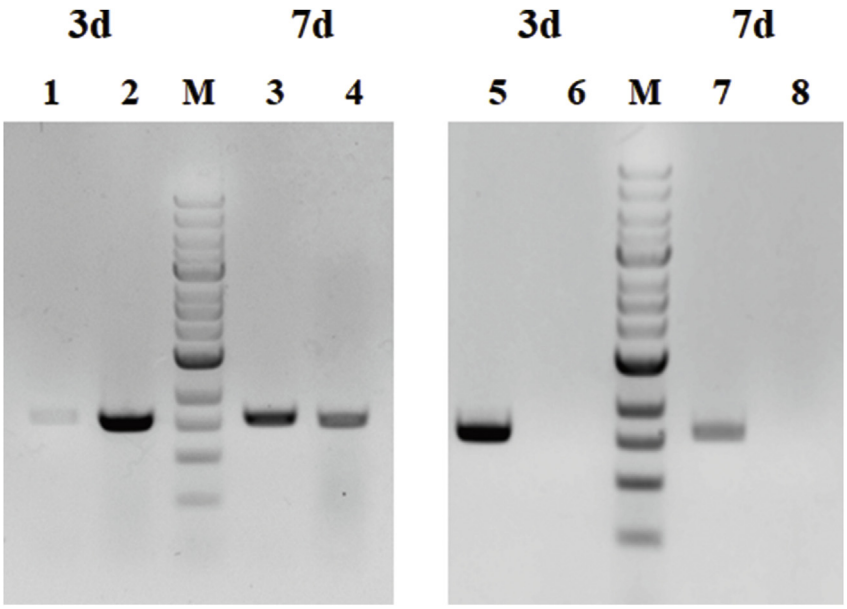

Fig. 3. Confirmation of rVHSV-GΔTM genome in EPC cells by RT-PCR. (A) EPC cells were infected with rVHSV-GΔTM at MOI 0.1, and the amplification of viral genome (a region ranging from G gene to NV gene) was analyzed from the supernatant (Lane 1, 3) and from cell pellets (Lane 2, 4) at 3 and 7 days post-infection. (B) To know whether the supernatant obtained from the 1st infection of EPC cells with rVHSV-GΔTM contained infective viruses, EPC cells were exposed to the supernatant and the amplification of viral was analyzed from the supernatant (Lane 5, 7) and from cell pellets (Lane 6, 8) at 3 and 7 days post-exposure (B).

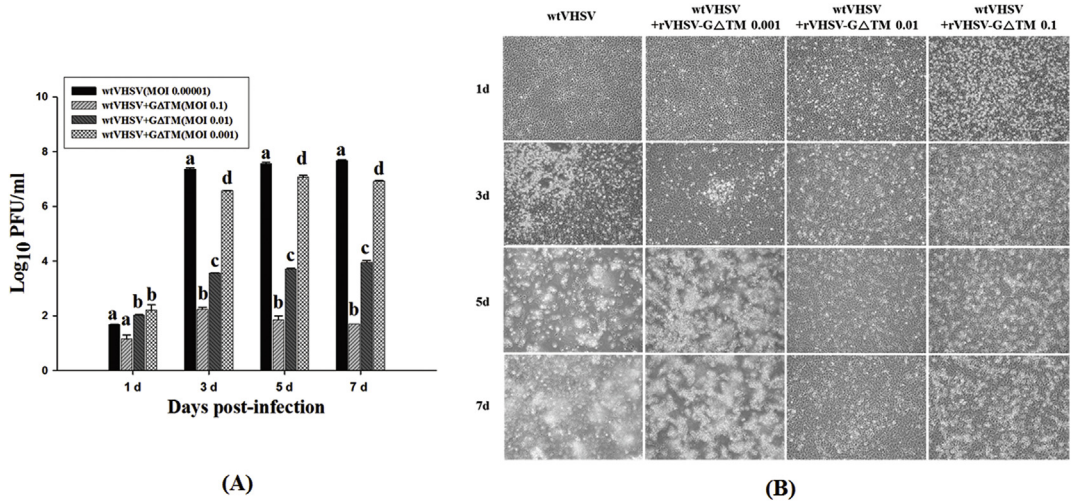

Fig. 4. Effect of rVHSV-GΔTM on the replication of wild-type VHSV in EPC cells. At 1, 3, 5, and 7 days post-infection, the virus titer (A) was analyzed by plaque assay and the progression of CPE (B) was observed. Different letters on the bars represent significantly different at $P < 0.05$.

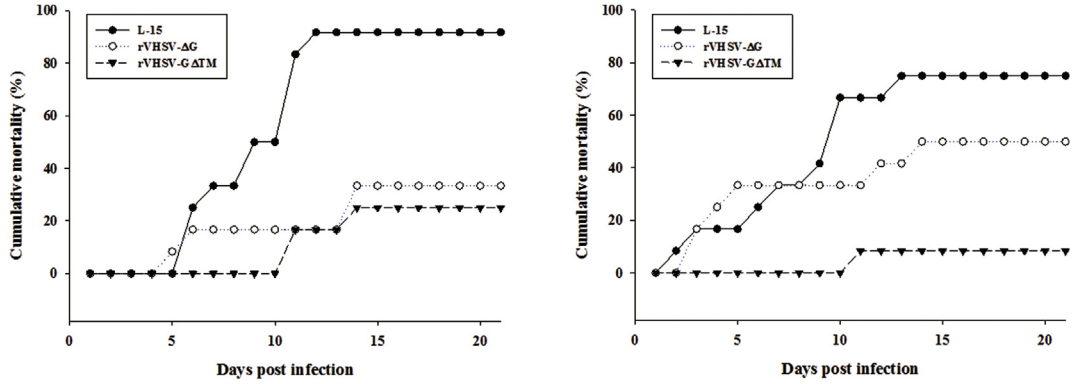

Fig. 5. Vaccine efficacy of rVHSV-GΔTM compared to rVHSV-ΔG. Olive flounder fingerlings were intramuscularly immunized with rVHSV-ΔG or rVHSV-GΔTM (2 replicates). Fish injected with L15 alone were used as a control group. After 4 weeks post-immunization, fish intramuscularly challenged with 1×10^4 PFU/fish of wild-type VHSV, and cumulative mortalities were recorded daily for 21 days post-infection.

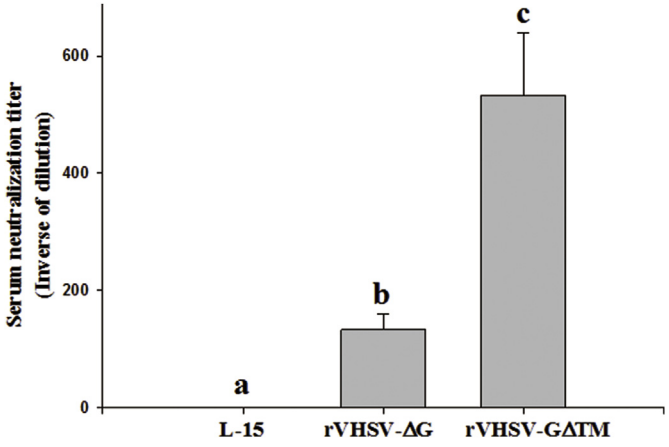

Fig. 6. Serum neutralization activity against wild-type VHSV. The sera of olive flounder immunized with rVHSV-ΔG or rVHSV-GΔTM were collected at 3 weeks post-immunization and used for neutralization test. Fish in control group were injected L15 alone. Values are mean ± standard error. Different letters on the bars represent significantly different at $P < 0.05$.

The induction of neutralizing antibodies is the key factor for the protection of fish from rhabdoviral diseases [13,14]. On the viral envelop, rhabdoviral G proteins usually present as trimeric forms, and the three-dimensional structure formed by the trimers can be important epitopes to induce protective immune responses [15,16]. Furthermore, it was reported that the truncated G protein lacking transmembrane region of rabies virus showed poor immunogenicity [17]. In this paper, however, as the G proteins produced in the cells by rVHSV-GΔTM are secreted out of cells by the deletion of transmembrane and C-terminal cytoplasmic region, the formation of the trimeric form might not be possible. While, in the present vaccine experiment, olive flounder immunized with rVHSV-GΔTM showed significantly higher titers of serum neutralization activity than fish immunized with rVHSV-ΔG suggesting that the G protein that is not only spiked on the viral envelop but also secreted extracellularly can contribute to the enhancement of adaptive humoral immunity.

Usually, the survival of vaccinated fish from VHSV challenges is highly related to serum neutralization activity [14]. In the present study, olive flounder immunized with rVHSV-GΔTM showed significantly higher survival rates than fish immunized with rVHSV-ΔG, suggesting that the amount of G protein provided to hosts is an important factor for the enhancement of vaccine efficacy against VHSV disease.

In a safety aspect, rVHSV-GΔTM could not replicate in infected cells, and, moreover, significantly inhibited the replication of wild-type VHSV when co-infected. This result suggests that the extracellularly secreted G proteins may compete with wild-type VHSV G proteins for cell surface receptors, and rVHSV-GΔTM would not worsen disease progression caused by wild-type VHSV infection.

In conclusion, the newly produced rVHSV-GΔTM can express higher amount of G proteins than rVHSV-ΔG, which made rVHSV-GΔTM more efficient than rVHSV-ΔG in inducing the protection of olive flounder against VHSV challenge.

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