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Protection against spring viremia carp virus (SVCV) by immunization with chimeric snakehead rhabdovirus expressing SVCV G protein

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Spring viremia of carp virus (SVCV) poses a significant threat to numerous cyprinid fish species, particularly the common carp (*Cyprinus carpio*), often resulting in substantial mortalities. This study explores the potential use of a chimeric recombinant snakehead rhabdovirus carrying the SVCV G gene (rSHRV-Gsvcv) as a live vaccine against SVCV infection. Through virulence testing in zebrafish at different temperatures (15 °C and 20 °C), no mortality was observed in groups infected with either rSHRV-wild or chimeric rSHRV-Gsvcv at both temperatures, whereas 100% mortality occurred in fish infected with wild-type SVCV. Subsequently, as no mortality was observed by rSHRV-Gsvcv, three independent experiments were conducted to determine the possible usage of chimeric rSHRV-Gsvcv as a vaccine candidate against SVCV infection. Fish were immunized with either rSHRV-Gsvcv or rSHRV-wild, and their survival rates against the SVCV challenge were compared with a control group injected with buffer alone at four weeks post-immunization. The results showed that chimeric rSHRV-Gsvcv induced significantly higher fish survival rates compared to rSHRV-wild and the control groups. These findings suggest that genetically engineered chimeric rSHRV-Gsvcv holds the potential for a prophylactic measure to protect fish against SVCV infection.

Key words: SVCV, chimeric rSHRV-Gsvcv, Live vaccine

Introduction

Viruses within the family *Rhabdoviridae* are responsible for considerable morbidity and mortality in vertebrates (Walker and Winton, 2010). In fish, several rhabdoviruses, such as spring viremia carp virus (SVCV), infectious hematopoietic necrosis virus (IHNV), and viral hemorrhagic septicemia virus (VHSV), have been designated as notifying pathogens by the World Organization for Animal Health (WOAH). Among them, SVCV lacks the NV gene in its genome, distinguishing it from IHNV and VHSV (Walker et al., 2021). SVCV belongs to the genus *Sprivivirus* and has five structural genes encoding nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L) (Ahne et al., 2002; Walker et al., 2022). SVCV has been recognized as the most lethal virus in cyprinid fish, especially common carp (Fijan, 1999), and has been classified as a notifiable disease in many countries, mandating the culling of infected and at-risk fish populations (Baudouy et al., 1980; Shao and Zhao, 2017). Although there have been reports on the prophylactic measures against SVCV (Ashraf et al., 2016), especially, the high effectiveness of DNA vaccines containing an SVCV G protein expression cassette (Embregts et al., 2017; Emmenegger & Kurath, 2008;

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Kanellos et al., 2006; Zhao et al., 2022), no commercial vaccine has been developed to date.

In a previous study, we generated a chimeric recombinant snakehead rhabdovirus (SHRV) by replacing the SHRV G gene ORF in the genome with the SVCV G gene ORF (rSHRV-Gsvcv) and analyzed its replication characteristics at different temperatures (Lee et al., 2021). SHRV, belonging to the genus *Novirhabdovirus*, was initially isolated from snakehead fish (*Ophicephalus striatus*) in Thailand (Wattanavijarn et al., 1986). Limited information exists regarding the virulence of SHRV in fish, with only a few studies reporting mortalities in adult zebrafish (*Danio rerio*) following intraperitoneal injection of SHRV (Alonso et al., 2004; Phelan et al., 2005). In the present study, we observed no mortality in adult zebrafish challenged with 1×10^4 pfu/fish of either wild-type SHRV or rSHRV-Gsvcv, whereas 100% mortality was observed following SVCV infection.

Live vaccines based on attenuated viruses offer advantages over inactivated or subunit vaccines due to their ability to induce robust protective immune responses similar to those elicited by natural viral infections (Choi et al., 2019; Kim and Kim, 2012; Kim et al., 2022). Non-virulent or weak-virulent viruses can be used as a delivery tool for antigens of virulent pathogens by generating chimeric viruses expressing heterologous antigens. Given the absence of mortality observed following rSHRV-Gsvcv infection, this study evaluated the vaccine potential of chimeric rSHRV-Gsvcv against SVCV through zebrafish immunization.

Materials and Methods

Cells 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. SHRV (ATCC-VR1386), rSHRV-wild, rSHRV-Gsvcv (Lee et al., 2021), and wild-type SVCV (K1 strain isolated from common carp in the year 2010 in Korea) were propagated in EPC cells in L-15 with 2% FBS and antibiotics at 28°C. Viral stocks were prepared by infecting a monolayer of EPC cells with the virus, followed by centrifugation of the supernatant when the extensive cytopathic effect (CPE) was observed. After filtration through a 0.45 μm pore size filter, the supernatant was aliquoted and stored at -80°C until further use.

In vivo virulence

Adult zebrafish (average weight 0.3 g) were randomly divided into 8 groups (16 fish/group) and acclimated at 15°C (4 groups) and 20°C (4 groups) for one week. Prior to virus challenge, fish were anesthetized with 100 ppm MS-222 (Sigma) and injected intramuscularly with L-15 alone (control group), rSHRV-wild, rSHRV-Gsvcv, or wild-type SVCV at 1×10^4 PFU/20 μl/fish. Mortalities were recorded daily for 21 days post-injection.

Quantitative Real-Time PCR (qRT-PCR)

Tissue samples from zebrafish (whole body except for the head and caudal fin) were homogenized with RiboEx (GeneAll, Korea) using a Tissue Lyser II (Qiagen), and then total RNA was extracted using a Hybrid-R kit (GeneAll). Subsequently, 1 μg of RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase containing random hexamer (Elpisbio, Korea). The resulting cDNA was used as a template for quantitative RT-qPCR. Each RT-qPCR reaction was conducted in a total volume of 20 μl containing SYBR green PCR master mix (Enzynomics, Korea), 1 μl of primer (each primer/5 pmol), 5 μl of template cDNA, and 3 μl of RNase-free water. RT-qPCR was carried out using a Light Cycler 480 (Roche) with the following protocol: initial denaturation at 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 20 sec. Primer sets used in the RT-qPCR analysis are listed in Table 1.

Table 1. Primers used for real-time qPCR

Primer name	Sequence (5′ → 3′)
For SHRV quantification	
SHRV_qPCR_F	CAGAACTTCCCCCCTAACG
SHRV_qPCR_R	CCTCCTGAAGGTTCTCTTGTG
For SVCV quantification	
SVCV_qPCR_F	ATCAGGCCGATTATCCTTCCA
SVCV_qPCR_R	AGATAAGCATTCACATGCTGTAT

Immunization and challenge

1) Experiment I

Zebrafish were divided into two groups with 2 replicates (15 fish/tank) and intramuscularly injected with L-15 alone (control group) or 1×10^4 PFU/20 μl/fish of rSHRV-Gsvcv. At 5 weeks post-immunization, fish were challenged with 1×10^3 PFU/fish of wild-type SVCV. The water temperature was maintained at 15 °C throughout the experiment, and mortality was monitored for 15 days.

2) Experiment II

Zebrafish were divided into two groups with 2 replicates (15 fish/tank) and intramuscularly injected with L-15 alone (control group) or 1×10^3 PFU/20 μl/fish of rSHRV-Gsvcv. At 4 weeks post-immunization, fish were challenged with 1×10^2 PFU/fish of wild-type SVCV. The water temperature was maintained at 15 °C throughout the experiment, and mortality was monitored for 21 days.

3) Experiment III

Zebrafish were divided into three groups with 2 replicates (10 fish/tank) and intramuscularly injected with L-15 alone (control group), 1×10^3 PFU/20 μl /fish of rSHRV-wild, or 1×10^3 PFU/20 μl/fish of rSHRV-Gsvcv. At 4 weeks post-immunization, fish were challenged with 1×10^3 PFU/fish of wild-type SVCV. The water temperature was maintained at 15 °C throughout the experiment, and mortality was monitored for 15 days.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, USA). Viral titers were analyzed using one-way ANOVA followed by Tukey HSD post-hoc test, with $p < 0.05$ was considered statistically significant. Survival kinetics data were analyzed using Kaplan-Meier survival analysis, and Log-rank tests (Mantel-Cox) were used to evaluate statistical significance ($p < 0.05$).

Results

in vivo virulence and viral titer

The virulence of rSHRV-wild, rSHRV-Gsvcv, and wild-type SVCV in zebrafish was assessed at 15°C and 20°C. Wild-type SVCV caused 100% mortality at both temperatures, whereas neither rSHRV nor rSHRV-Gsvcv induced mortality at either temperature (Fig. 1a).

On the final day of the challenge experiment, the presence of each virus in zebrafish was determined by quantitative RT-qPCR using samples from three randomly selected fish per group. High-titer SVCV was detected in dead fish at both 15°C and 20°C (6.7×10^6 copies/mg and 6.7×10^6 copies/mg, respectively). As for rSHRV-wild and rSHRV-Gsvcv, low-titer were detected in surviving fish at both temperatures (8.2×10^3 copies/mg, 5×10^3 copies/mg, respectively at 15°C and 2×10^3 copies/mg, 3×10^3 copies/mg, respectively at 20°C) (Fig. 1b). The titer of rSHRV-wild at 15°C (8.2×10^3 copies/mg) was significantly higher than that at 20°C (2×10^3 copies/mg) (Fig. 1b).

Immunization and challenge

In experiment I, fish immunized with 1×10^4 pfu/fish of rSHRV-Gsvcv exhibited significantly higher survival rates following SVCV challenge (Fig. 2a). In experiment II, a tenfold lower titer of rSHRV-Gsvcv (1×10^3 pfu/fish) also led to significantly higher survival rates (Fig. 2b). In experiment III, fish immunized with 1×10^3 pfu/fish of rSHRV-wild showed

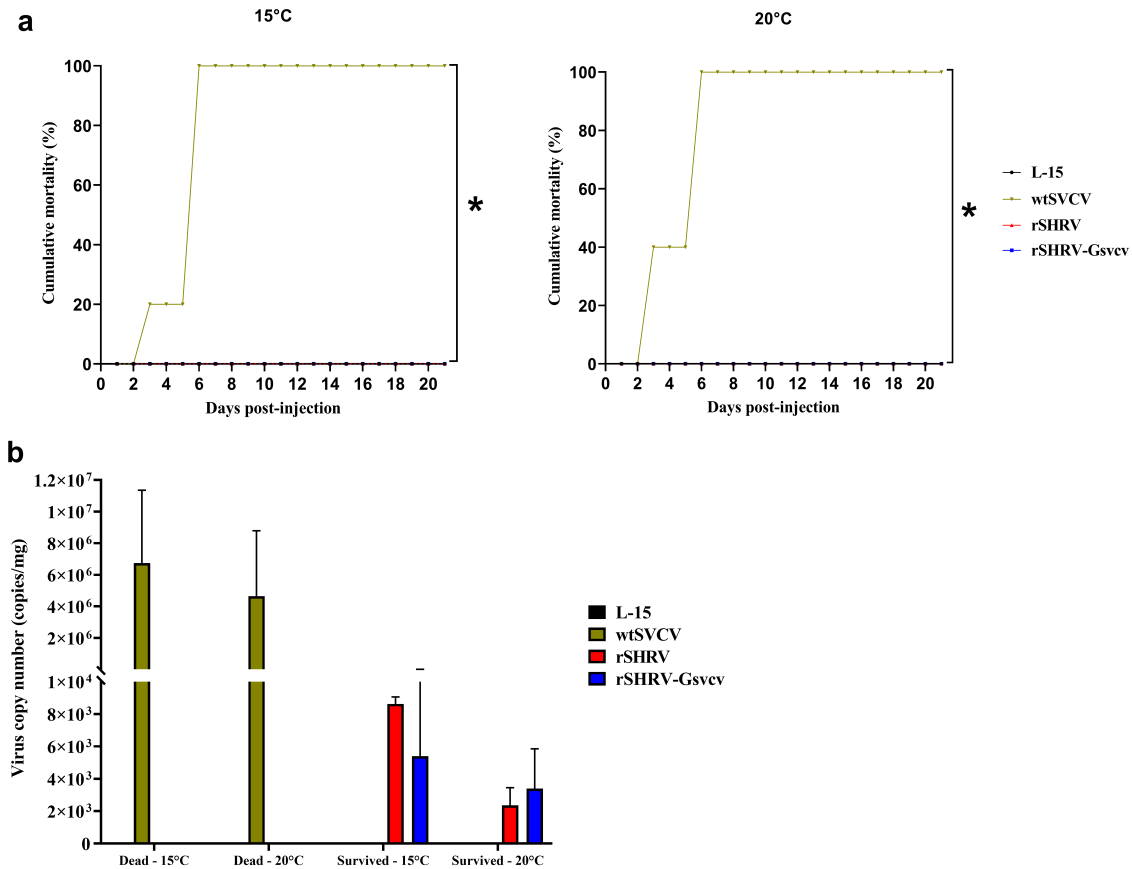

Fig. 1. Virulence of chimeric rSHRV-Gsvcv in zebrafish at 15°C and 20°C. (a) Cumulative mortality of zebrafish injected with control viruses (wild-type SVCV, wtSVCV; rSHRV-wild, rSHRV) and the chimeric rSHRV-Gsvcv. (b) Quantification of the chimeric rSHRV-Gsvcv from zebrafish in comparison with wild-type SVCV (wtSVCV) and rSHRV-wild (rSHRV). The asterisk represents statistically significant at $p < 0.05$.

90-100% mortality by SVCV challenge, which was similar to the mortality of the control group (100%), whereas fish immunized with 1×10^3 pfu/fish of rSHRV-Gsvcv showed 10-20% mortality (Fig. 2c).

Discussion

Zebrafish has been used as an in vivo model fish on many occasions involving the study of rhabdoviruses, including SVCV and SHRV (Sanders et al., 2003). In the present study, SVCV induced high mortality in zebrafish, whereas rSHRV-Gsvcv did not lead to infection-mediated mortality. The glycoprotein of rhabdoviruses plays a crucial role in viral infection, which has led to the development of glycoprotein-based vaccines (Kim et al., 2023; Martinez-Lopez et al., 2014; Puente-Marin et al., 2018). In our investigation, fish immunized with rSHRV-Gsvcv exhibited over 80% survival rates, contrasting with fish immunized with rSHRV, which experienced 90-100% mortality. This suggests that the SVCV G gene in the chimeric virus was the primary factor contributing to the high protection.

Previously, Emmenegger et al. (2018) assessed the in vivo virulence of a chimeric rIHNV-Gsvcv to rainbow trout (*Oncorhynchus mykiss*), common carp

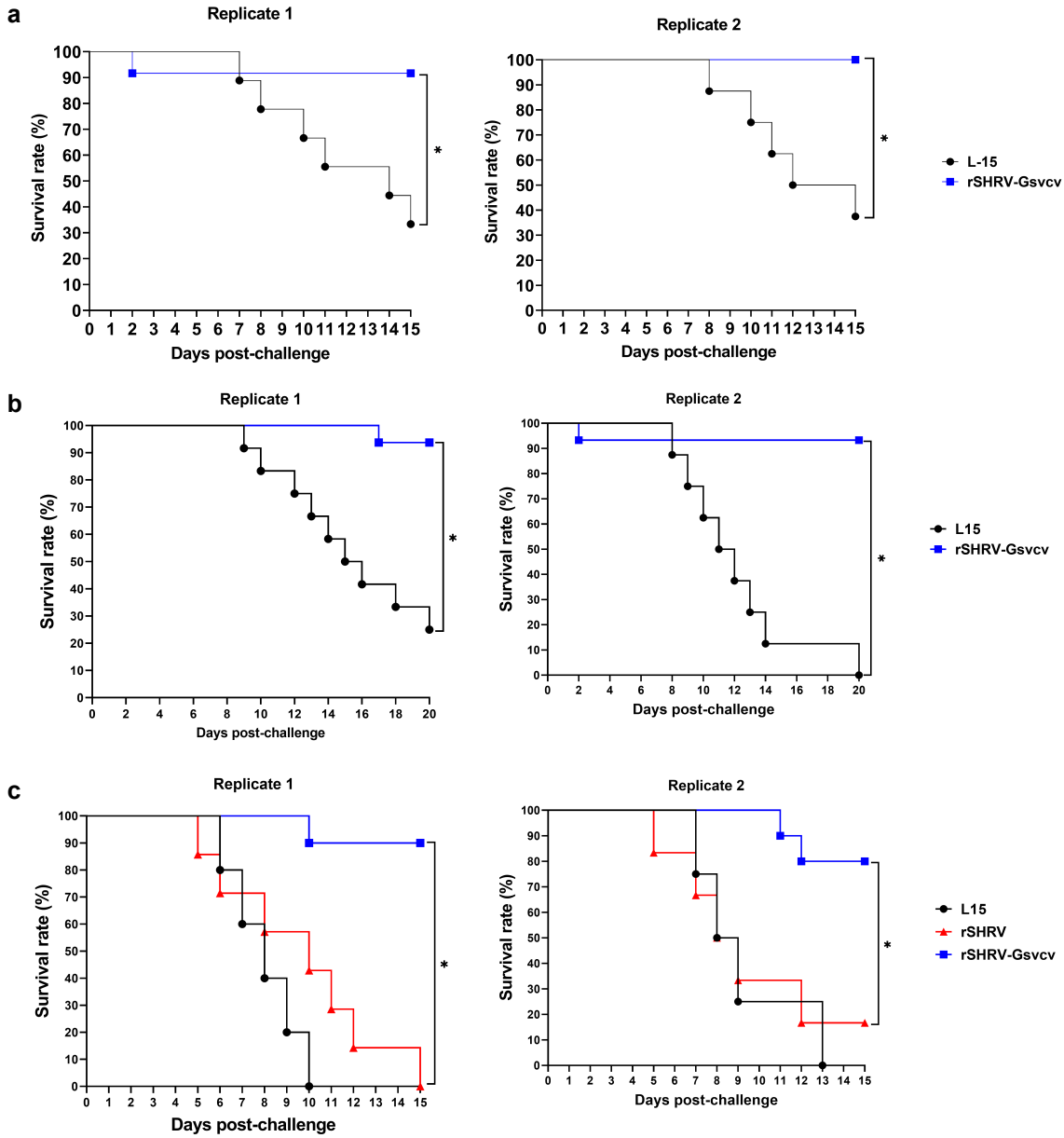

Fig. 2. Protective effect of chimeric rSHRV-Gsvcv against SVCV infection. (a) and (b) represent the survival rate obtained from experiment I and experiment II, respectively, showing the safety and protective effect of the chimeric virus depending on the dose. (c) represents the survival rate of the chimeric rSHRV-Gsvcv compared to a control virus rSHRV-wild. The asterisk represents statistically significant at $p < 0.05$.

(*Cyprinus carpio carpio*), and koi (*Cyprinus carpio koi*) that were acclimated to 10°C. They found that rIHNV-Gsvcv induced high mortality only in rainbow trout but did not induce mortality in common carp and koi. Furthermore, koi that survived rIHNV-Gsvcv infection exhibited strong resistance against virulent SVCV infection, suggesting the possible use of rIHNV-Gsvcv as a prophylactic vaccine. However, the high

virulence of the IHNV-based chimeric virus in rainbow trout may pose challenges in using chimeric IHNVs as live vaccines against heterologous pathogens.

Although reports on the virulence of SHRV are limited (Phelan et al., 2005), in our preliminary experiments, we observed no mortality in carp, koi, and even snakehead fingerlings by SHRV infection. Therefore, SHRV is considered safer than other fish rhabdoviruses as an antigen-delivery tool in fish through the generation of recombinant chimeric viruses. Additionally, as SHRV can replicate in a wide temperature range, its usage can be more versatile compared to other fish novirhabdoviruses that replicate only at low temperatures. Further investigation into possible host ranges is necessary to expand the availability of chimeric SHRVs.

Despite the high protectivity observed in the immunization experiments in this study, further studies are needed to elucidate the immunological phenomena induced by the chimeric SHRV. Moreover, as the present chimeric SHRV retains replicative ability in host cells, the possibility of inducing pathological symptoms in infected fish cannot be disregarded. The significantly higher titer of rSHRV-wild at 15°C compared to 20°C suggests that reduced immunity of zebrafish due to low temperature may facilitate better replication of SHRV, despite its optimal temperature being over 20° C. Therefore, safer forms such as single-cycle chimeric SHRVs and alternative administration routes should be considered to enhance the likelihood of obtaining approval as practical vaccines.

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