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DNA vaccine protects ornamental koi (*Cyprinus carpio koi*) against North American spring viremia of carp virus

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ABSTRACT

The emergence of spring viremia of carp virus (SVCV) in the United States constitutes a potentially serious alien pathogen threat to susceptible fish stocks in North America. A DNA vaccine with an SVCV glycoprotein (G) gene from a North American isolate was constructed. In order to test the vaccine a challenge model utilizing a specific pathogen-free domestic koi stock and a cold water stress treatment was also developed. We have conducted four trial studies demonstrating that the pSGnc DNA vaccine provided protection in vaccinated fish against challenge at low, moderate, and high virus doses of the homologous virus. The protection was significant ($p < 0.05$) as compared to fish receiving a mock vaccine construct containing a luciferase reporter gene and to non-vaccinated controls in fish ranging in age from 3 to 14 months. In all trials, the SVCV-G DNA immunized fish were challenged 28-days post-vaccination (546 degree-days) and experienced low mortalities varying from 10 to 50% with relative percent survivals ranging from 50 to 88%. The non-vaccinated controls and mock construct vaccinated fish encountered high cumulative percent mortalities ranging from 70 to 100%. This is the first report of a SVCV DNA vaccine being tested successfully in koi. These experiments prove that the SVCV DNA (pSGnc) vaccine can elicit specific reproducible protection and validates its potential use as a prophylactic vaccine in koi and other vulnerable North American fish stocks.

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1. Introduction

Spring viremia of carp virus (SVCV) is a rhabdoviral pathogen that frequently decimates common carp (*Cyprinus carpio carpio*) stocks throughout Europe [1,2]. Carp populations in the European countries of Russia, Romania, Netherlands, Moldavia, Georgia, Germany, France, United Kingdom, and Denmark have the highest reported prevalence [3]. In fish species that succumb to infection by SVCV, the spleen, kidney, intestines, and air bladder are typically inflamed, hemorrhaging, or swollen. Disease progression leads to necrosis of the internal organs and eventually death. Outbreaks at common carp farms in Europe normally occur in the spring, as the water temperature begins to rise after a cold winter period. The highest fish mortalities due to SVCV infection occur between 11 and 17 °C [4,5]. Common carp belonging to the *Cyprinidae* family are the principal host species of SVCV [6]. Natural infections of SVCV have also occurred in other cyprinid fish including koi (*Cyprinus carpio koi*), goldfish (*Carassius auratus*), crucian carp (*Carassius carassius*), silver carp (*Hypopthalmichthys molitirix*), bighead carp (*Aristichthys nobilis*), grass carp (*Ctenopharyngodon idella*), orfe (*Leucisucus idus*), and tench (*Tinca tinca*) [6,7]. Experimental infection of other cyprinid species included roach (*Rutilus rutilius*) [8], zebrafish (*Danio rerio*) [9], and fathead minnow (*Pimephales promelas*) [Emmenegger unpublished data]. Fish species from other families of *Poeciliidae*, *Esocidae*, *Centrarchidae*, *Siluridae*, and *Salmonidae* have also been infected by SVCV [2,10]. Due to the highly infectious nature of SVCV and potential impact this virus could have on susceptible fish populations globally, any detection of SVCV requires notification within 48 h to the Office of Internationale Epizootic (OIE), the organization charged with regulating world animal health. SVCV is one of only nine piscine viruses recognized worldwide by the OIE as a notifiable animal disease.

In April of 2002, at one of the largest koi production facilities in the United States, yearling koi in one pond began dying from SVCV [11]. Subsequently the virus was detected in other ponds at the facility, 15,000 fish died from SVCV and another 135,000 fish were euthanized from ponds located both in North Carolina and Virginia [12]. One month later in an apparently unrelated incident, dead wild carp began washing up on the shores of a Wisconsin lake. Mortalities reached 1500 and the causative agent of the epidemic was SVCV [13]. One year later the virus was isolated from a healthy common carp during a fish health screening in an Illinois water channel that is linked to Lake Michigan. In 2004 there were two outbreaks of SVCV, one at a private koi pond in Washington State

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and the other at a commercial koi hatchery in Missouri [14]. In June 2006, SVCV was found for the first time in Canada in common carp from Lake Ontario [15]. These fish were scheduled for shipment to France, but the virus was detected during an exportation disease screening. Later in October 2006 the United States Department of Agriculture (USDA) instituted regulations restricting the importation of live fish, fertilized eggs, and gametes of specific fish species susceptible to SVCV. Until the first outbreak in 2002, SVCV had never been reported in North America. Six isolations of this exotic virus in the past 6 years and the import restrictions placed on SVCV susceptible fish are warnings of the potential invasiveness and impact SVCV could have on vulnerable fish stocks in North America.

Eradication of SVCV infected fish and hygiene measures are the standard methods used to combat SVCV [2,16,17]. Therapeutic and preventative strategies to control SVCV have been ineffective and as such there is no commercially available SVCV vaccine. There have been previous reports of inactivated SVCV vaccines using European strains providing limited protection [18–22]. However, continued research on the inactivated SVCV vaccine has not been pursued in part due to the risks associated with incomplete activation of the virus, cumbersome legal and marketing restrictions, prohibitively expensive production costs, and the lack of a quantitative assessment of the protection levels provided by the vaccine [17,23].

DNA vaccines targeted against viral pathogens are an attractive alternative to traditional vaccines (i.e., inactivated, attenuated or protein subunits) for a variety reasons: straightforward design and construction, heat stability, low production costs, and long-term storage capabilities [24,25]. In addition, there is no risk of reversion to a pathogenic form and they have virtually no chemical impurities [26]. Hurdles currently being addressed for DNA vaccines include regulatory approval, promoter selection, and delivery technologies. Despite these concerns research on DNA vaccines fighting fish pathogens has increased steadily for the last 10 years [27]. Previous SVCV DNA vaccines [28] designed against European SVCV isolates have demonstrated lower efficacy as compared to fish DNA vaccines against infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) [25]. All three viruses, SVCV, IHNV, and VHSV, belong to the rhabdovirus family, but are separated into two different genera [29]. IHNV and VHSV belong to the *Novirhabdovirus* genus of viruses whose genome contains five structural genes, N, M, P, G, and L, and a sixth nonvirion (NV) gene of indeterminate function. SVCV, which has tentatively been assigned to the *Vesiculovirus* genus, lacks the NV gene [29,30]. Phylogenetic analysis of a partial SVCV G-gene region divided isolates into four genogroups, enabling SVCV isolates from Europe (Genogroups Ib, Ic, and Id) to be distinguished from those originating from Asia (Ia) [44]. Further phylogenetic analyses revealed that all six SVCV isolates detected in North America clustered in the SVCV Ia genogroup, suggesting that the isolates were of Asian origin [14,15]. The G-gene of these fish rhabdoviruses codes for the surface glycoprotein, which is the primary antigen that the fish host mounts an immune response against and is the target gene used in the DNA vaccine constructs. The novirhabdovirus DNA vaccines have demonstrated long-lasting protection with small doses in a variety of salmonid species, against various viral strains [27,31,32]. In 2005 the IHNV DNA vaccine was licensed in Canada for use in the Atlantic salmon aquaculture industry and the VHSV DNA vaccine has undergone field trials at rainbow trout farms in Denmark [24,32].

To date attempts to develop an equally efficacious SVCV DNA vaccine have not been successful. Early reports with European genogroup SVCV DNA vaccines from a Russian laboratory suggested some efficacy, but have not been confirmed (reviewed in [25]). More recently mixtures of 10 SVCV DNA vaccine plasmids containing partial or complete G gene fragments from the European SVCV reference strain (Fijan-Genogroup Id) have been tested in carp [28]. The majority of treatment groups had little protection, with RPS values of −11 to 33%. One group of fish receiving a combination of three plasmids had an RPS of 48% in a single trial, but the specific plasmid responsible for protection was not identified.

The presence of SVCV in the US and Canada has renewed research efforts to develop an effective DNA vaccine to prevent the spread and establishment of SVCV in North America. In this project, a novel SVCV DNA vaccine utilizing the North Carolina (nc) SVCV G-gene was designed and tested in four trial experiments. In order to test the vaccine a reliable challenge model was developed by testing the susceptibility of different fish host species to the North American SVCV and devising challenge treatments that induced rapid and reproducible infections in the host.

2. Materials and methods

2.1. Fish stocks

Goldfish (*C. auratus*) less than 1 year old and 7–10 cm in length were shipped from a facility, that meets the OIE standard as a compartment free of SVCV, to the wet laboratory facility at the Western Fisheries Research Center (WFRC, Seattle, Washington). Fish were housed in tanks with flow-through sand-filtered and UV-treated fresh water at water temperatures of 16–18 °C. Fish were monitored daily and fed every other day Wardley Ten floating pellets (Hartz Mountain Co.). Goldfish were maintained in the stock tanks until commencement of the SVCV susceptibility challenges.

Koi (*Cyprinus carpio koi*) from a specific pathogen-free domestic stock were obtained from a local koi farm (Pan Intercorp., Kenmore, Washington). The koi distributor annually breeds his own koi stock. The breeder has voluntarily participated in the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) screening program for SVCV in ornamental fish. One-month-old specific pathogen-free domestic koi were transferred to the wet laboratory facility at the WFRC and reared under the same conditions described for the goldfish. Koi were also fed every other day, but received a mixed feed diet of moist and dry pellets consisting of Life Stage Diet Food (Oregon Biodiet), Wardley Pond 10 (Hartz Mountain Co.), and Hikari Gold (Kyorin Food Industries). The amount, pellet size, and type of feed varied as the koi aged. Koi were held in stock tanks until initiation of the susceptibility challenges, cold stress challenge experiment, or vaccine trial studies.

2.2. Virus propagation

The North American SVCV isolate from North Carolina (SVCVnc) was sent by Dr. Andy Goodwin, the first scientist to isolate and identify SVCV in North America [11]. The SVCVnc isolate was propagated in an *epithelioma papulosum cyprini* (EPC) cell line [33] at a constant temperature of 20 °C in minimum essential medium (MEM; Invitrogen Inc.) supplemented with 10% fetal bovine serum (Hyclone Inc.) and 2 mM L-glutamine (Invitrogen Inc.), and buffered to pH 7.0 with 7.5% sodium bicarbonate (Fisher Scientific Co.). Virus titers for the challenge inocula were determined by plaque assay following the procedure outlined by Batts and Winton [34] with a modified incubation temperature of 20 °C.

2.3. Development of the challenge model

2.3.1. Susceptible host species challenges

Prior to challenge fish were transferred to an aquatic biosafety level 3 (BSL-3) laboratory, housed in a separate building from the main WFRC wet laboratory. Due to OIE listing of SVCV and its exotic

pathogen status in North America all *in vivo* challenges adhered to aquatic BSL-3 containment regulations. After transfer to the BSL-3 at 16–18 °C, fish in this study were subjected to a slow cold water stressor in which water temperature was decreased 1 °C daily until tank water temperatures reached 10–12 °C. Fish were then acclimated at this colder temperature for an additional 5 days.

For the injection challenge, fish were anaesthetized by immersion in 100 μg/ml of tricaine methane sulfonate (MS-222; Argent Chemical Laboratories) buffered with sodium bicarbonate to a pH of 7.5. All injection-challenged fish received a 100 μl volume of viral supernatant via an intraperitoneal injection (IP) for a total of 1×10^6 PFU per fish. After injection fish were transferred to a recovery tank until consciousness and swimming mobility were regained. A mock injection challenge of an equivalent volume of MEM-10 media was also included for all susceptibility experiments. Fish challenged at various virus concentrations by immersion were held in a volume of 3 l for 1 h with aeration after which intake water flow was resumed. Mock immersion challenges occurred under the same conditions, but an equivalent volume of MEM-10 culture medium was added into the water instead of virus. All mock and virus challenge treatments were done in duplicate tanks. Injection and immersion challenges were observed for 30 days and fish were fed every other day during the challenge. Dead fish were removed daily and mortalities were recorded. Fisher's exact test was performed to confirm that there was no statistically significant difference between mortalities occurring in the duplicate treatment tanks. Average cumulative percent mortality (CPM) was calculated as the number of fish that died from the pooled duplicate tanks divided by the total number fish receiving that treatment × 100.

Goldfish of an average weight of 14.6 g were exposed to SVCVnc in two susceptibility experiments. Each experiment consisted of immersion challenges at virus doses of 1×10^3, 1×10^4, and 1×10^5 PFU/ml, and one injection challenge with each fish receiving 1×10^6 PFU. There were 10 goldfish in each duplicate treatment group for each experiment.

One-month-old koi weighing on average 0.94 g were also challenged with SVCVnc in two experiments. The first preliminary experiment challenged three koi in each duplicate group by immersion at 1×10^5 PFU/ml or by injection with 1×10^6 PFU/fish. The second experiment used the same challenge protocols and doses, but with duplicate treatment groups of 10 koi each. Two koi each from the immersion and injection challenge treatment groups from the second experiment were titered for virus. Virus quantification in whole fish homogenate samples was done by plaque assay following the methods described by Batts and Winton [34]. Virus titers were calculated as the logarithmic geometric mean of the virus concentration in PFU/g for virus-positive fish in each treatment group.

2.3.2. Cold stress challenges in koi

The mortality outcome of koi subjected to two different cold water temperature stressors prior to virus challenges were compared. It has been reported that exposure of fish to a cold temperature stress treatment prior to SVCV challenge is important for disease initiation [4]. One challenge protocol used the cold stressor that was described previously in the species susceptibility testing, in which fish were slowly acclimated to colder water temperatures (10–12 °C) and held at that temperature for several days. This slow acclimation to colder water temperatures takes approximately 14 days before the challenge can be initiated. The other treatment tested was a rapid cold water stressor, in which fish were transferred on the same day of challenge from stock water temperatures of 16–18 °C to challenge temperatures of 10–12 °C. Duplicate groups of 10 koi at 3.5 months of age and weighing 3.5 g were subjected to each cold water stress treatment. Fish were then challenged either by immersion or injection using the same challenge doses and methods describe for koi in the susceptibility experiments. After challenge the BSL-3 laboratory water temperature was raised 0.5 °C a day until the temperature reached 14 °C and remained at that temperature for the duration of the experiment. The CPM and the mean day to death (MDD) were the parameters used to compare cold water stress treatments. MDD was the sum of the number of days post-challenge that each fish died on divided by the total number of mortalities for a particular treatment. A proportion of the survivors and dead fish were screened for virus by plaque assay following the previously cited protocol.

2.4. Vaccine production and testing

2.4.1. Vaccine construction

The entire glycoprotein (G) gene of the SVCV from North Carolina was sequenced (Genbank AY527273 by Emmenegger in 2004) following the protocols previously described by Emmenegger [35], utilizing conserved G-gene primers designed against European SVCV strains [30,36,44]. The North Carolina (nc) SVCV G-gene was selected as the target gene for vaccine construction, since it most closely matched the consensus G-gene sequence of the five U.S. SVCV isolates (data not shown). Total RNA from SVCVnc viral stock was extracted with Trizol reagent (Invitrogen Inc.) according to manufacturer's instructions and resuspended in 50 μl of enzyme-grade water.

Specific SVCVnc G-gene forward (5′-CACCATGTCTATCATCAGCTACATC-3′) and reverse (5′-CTAAACGAAGGACCGCATTTCGTG-3′) cloning primers were designed to facilitate directional cloning of the cDNA generated by reverse transcription (RT) and polymerase chain reaction (PCR), following the procedures described by Emmenegger [37]. The blunt-end amplified products were directionally cloned into the pcDNA3.1 vector using the TOPO expression kit (Invitrogen Inc.) following the manufacturer's instructions. Transformed clones with the targeted insert were selected by the rapid colony PCR protocol (Novagen) utilizing vector primers (T7 and reverse BGH) (Invitrogen), and internal SVCV G-gene primers 379+ (5′-TTTCCCCCTCAAAGTTGCGG-3′) and 978-/F1(5′-TCTTGGAGCCAAATAGCTCARRTC-3′). A restriction enzyme digest was performed to confirm the correct size and orientation of the inserts (data not shown). One clone was selected for further vaccine development and henceforth will be referred to as pSGnc (SVCV DNA) vaccine. The entire G-gene nucleotide sequence of the pSGnc construct was determined to verify that no sequence changes had occurred during RT/PCR and cloning. The control mock vaccine (pLuc) containing the luciferase reporter gene in the pCDNA-3.l vector was produced earlier by Corbeil et al. [31,38]. Both plasmid constructs were propagated in *Escherichia coli* and purified by an alkaline lysis methodology described by Saporito-Irwin et al. [39], as used previously with the IHNV DNA vaccine [31].

2.4.2. Vaccination

In each of the four trial experiments (A–D) there were three treatment groups: the SVCV DNA vaccine (pSGnc), the mock luciferase vaccine (pLuc), and a non-vaccinated (NV) control. The NV control treatment group fish received no injection and were transferred from the stock tanks to a separate holding tank receiving the same water source as the injected fish. Average fish weights and ages at vaccination for the four trials are shown in Table 3. Prior to vaccination all fish were anaesthetized as previously described. Each fish received a 10 μg vaccine dose in a 50 μl volume of phosphate buffered saline (PBS) via an intramuscular (IM) injection in the epaxial muscle midway between the posterior end of the dorsal fin and lateral line. The delivery volume was selected based on IHNV

DNA vaccine studies in which juvenile rainbow trout (*Oncorhynchus mykiss*) weighing 1.5 g are typically vaccinated with a 50 μl volume of vaccine (31). After recovery, all fish from each treatment group were transferred to separate holding tanks at a water temperature of 19–20°. The slightly warmer temperature after vaccination was used in an effort to enhance immune response to the vaccine. Vaccinated fish were monitored daily and fed every other day.

2.4.3. Virus challenge after vaccination

Twenty-eight days [approximately 546 degree-days (°D)] post-vaccination fish were transferred to the aquatic biosafety level 3 (BSL-3) laboratory. All koi were subjected to a rapid cold water stressor (i.e., transfer from stock water temperatures of 19–20 °C to challenge water temperatures of 10–12 °C) prior to virus challenge. For viral challenge, fish were injected IP with 100 μl of viral supernatant after anaesthetizing the fish as previously described. The homologous virus strain (SVCV North Carolina) that was utilized in the DNA vaccine construct was used in all challenges. Challenge doses were shown in Table 3. A mock challenge, as previously described, was also included for each treatment group for all trial experiments. Each mock and viral-challenged treatment group was divided into duplicate groups of 10 fish/tank. The BSL-3 laboratory water temperature on the day of challenge was 10–12 °C and was raised 0.5 °C a day until the temperature reached 14 °C. The challenge conditions of each trial are outlined in Table 3.

Fish health was monitored daily and fish were fed every other day for a 26–29 day observation period. Dead fish were removed from the tank, and stored at −80 °C. Efficacy of the SVCV DNA vaccine was assessed by comparing the cumulative percent mortality (CPM), and relative percent survival (RPS) between treatment groups. RPS values were calculated using the following formula: [1 − (CPM of vaccinated group/CPM of the negative control)] × 100 [40]. Virus titers of sampled dead fish and survivors, and the mean day to death of the mortalities from each of the treatment groups were also used to evaluate vaccine performance. Fisher's exact test was used to confirm that there was no statistically significant difference between mortalities in the duplicate treatment tanks. Survival curves were calculated by Kaplan-Meier analysis to assess differences between treatment groups. The Mantel-Cox log-rank test determined if the resultant survivor curves were significantly different (Systat 8.0). Forty-five percent of the mortalities from trials A and B, and all mortalities from trials C and D were screened for virus. A proportion of the survivors were also sampled for virus quantification. Virus quantification of whole fish samples were processed blind by plaque assay as previously described.

3. Results

3.1. Susceptibility testing of goldfish and koi

For development of a reliable challenge model to test the SVCV DNA vaccine, koi and goldfish were compared for susceptibility to SVCVnc by injection and immersion challenges. The results are summarized in Table 1. Overall, the majority (96%) of the goldfish exposed to SVCVnc survived both challenge experiments. All koi died after the SVCVnc injection challenge and 75% of the koi succumbed after immersion challenge in the second koi susceptibility experiment. Confirmation of this domestic koi stock's susceptibility to SVCVnc validated its usage as the positive control fish species in virus challenges and as a potential cyprinid host for SVCVnc vaccine development.

3.2. Cold stress challenges in koi

There was no significant difference in mortality between the two cold water stress treatments that the koi underwent prior to virus challenge (Table 2). The cumulative percent mortalities were identical between the two stress treatments for both immersion (75% CPM) and injection (100%) challenges. Among mock challenged fish the single fish that died and all the survivors tested had no

Table 1
Development of challenge model: selection of fish species and SVCVnc challenge conditions

Challenge method and dose[a]	Goldfish mortality[b]		Koi mortality[b]	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Immersion: 1×10^3 PFU/ml	1/20	0/20	nt	nt
Immersion: 1×10^4 PFU/ml	0/20	0/20	nt	nt
Immersion: 1×10^5 PFU/ml	1/20	1/20	4/6	15/20
Mock immersion	0/20	0/20	0/6	0/20
Injection (IP): 1×10^6 PFU	4/20	0/20	6/6	20/20
Mock injection	2/20	0/20	0/6	0/20

Based on these results, koi challenged with an intraperitoneal dose of 10^6 PFU or baths at 10^5 PFU/ml were chosen for further evaluation.

[a] Goldfish (*Carassius auratus*) and koi (*Cyprinus carpio koi*) were challenged with SVCVnc either by water bath immersion at the listed virus titer or intraperitoneally (IP) injected with 1×10^6 PFU. Mock challenged fish were exposed to culture media (MEM-10-SB).

[b] The mortality ratio listed is the number of dead fish over the total number of fish challenged in pooled data from duplicate tanks. "nt" indicates not tested.

Table 2
Development of challenge model: the effect of two thermal stress profiles on mortality of koi challenged with SVCV

Challenge method and dose[a]	Slow cold water stressor[b]		Rapid cold water stressor[b]	
	Mortality (%)	MDD	Mortality (%)	MDD
Mock injection	5	0	0	N/A
Mock immersion	0	N/A	0	N/A
Immersion: 1×10^5 PFU/ml	75	18	75	17
Injection: 1×10^6 PFU	100	5	100	6

Based on these results, the rapid cold stressor was applied in subsequent SVCV challenges since there was no difference in mortality between the two thermal profiles and the rapid stressor was more time efficient.

[a] Koi were challenged with SVCVnc either by water bath immersion or intraperitoneally injection. Mock challenged fish were exposed to culture media (MEM-10-SB).

[b] The slow cold water stressor profile slowly acclimated koi from stock water temperatures of 16–18 °C to colder water temperatures (10–12 °C) over a 2-week period prior to virus challenge. The rapid cold water stressor profile consisted of transferring koi the same day of virus challenge from the stock water temperatures (16–18 °C) to water challenge temperatures (10–12 °C).

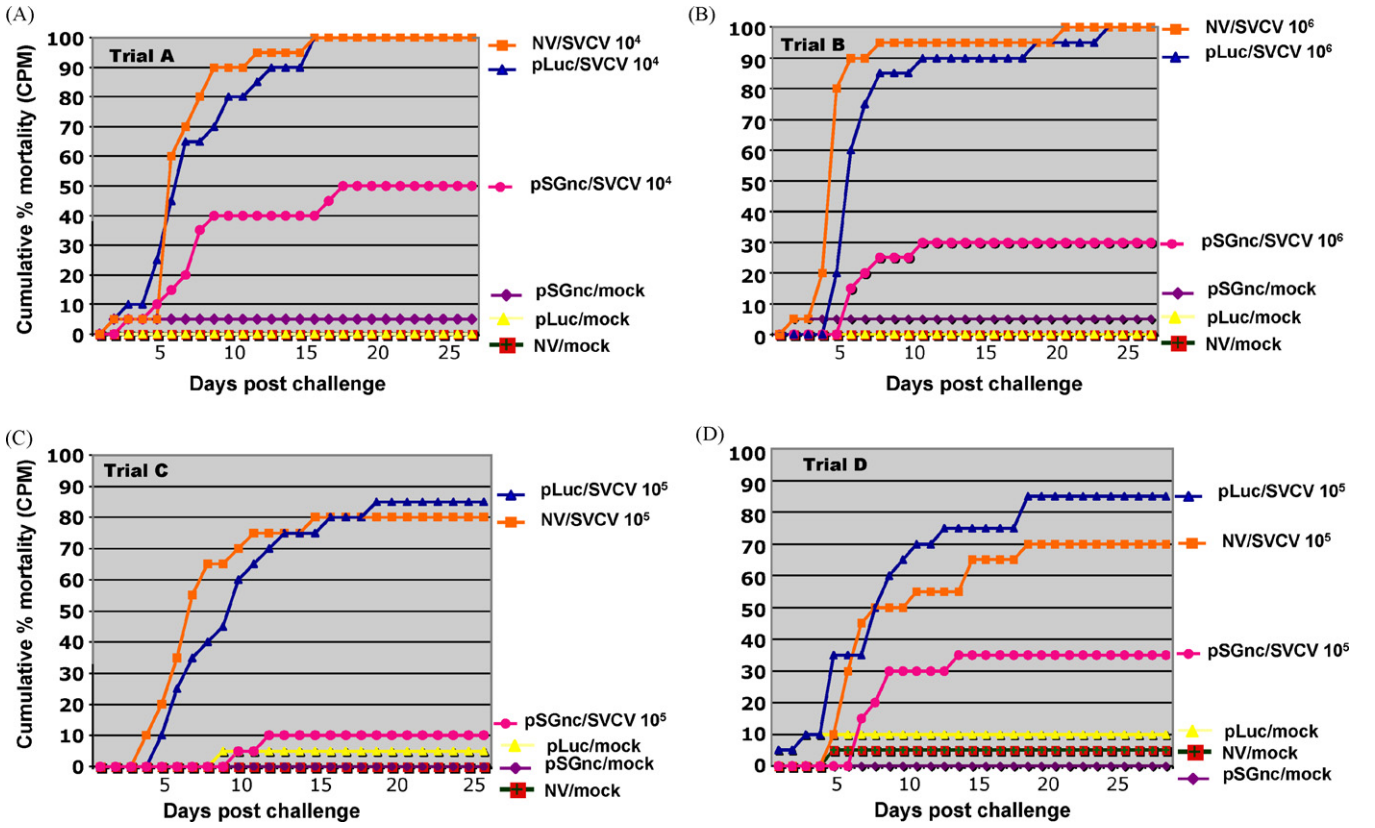

Fig. 1. Cumulative mortality of vaccinated koi following challenge with SVCVnc. Koi were challenged 28 days (546 °D) after pSGnc vaccination using different virus concentrations in trials A–D. Mortality was recorded daily during a observation period ranging of 26–29 days. Data shown is the average from duplicate groups for each treatment.

detectable virus ($n = 9$). From the virus-challenged koi all the sampled dead fish and survivors ($n = 19$) tested positive for virus with high virus titers that were nearly all (18/19) above 1×10^6 pfu/g. The rapid cold stressor was adopted into the SVCV challenge model protocol since it was more efficient and produced the same mortalities as the slow acclimation cold stressor.

3.3. Vaccine trials

The mock challenged fish experienced negligible mortalities ranging from 0 to 10% in all four trials (Fig. 1). The small number of mock-challenged mortalities showed no clinical signs of disease and those fish screened for virus were negative.

In all four vaccine trials non-vaccinated (NV) fish exposed to virus suffered mortalities ranging from 70 to 100% (Fig. 1, Table 4). The virus challenged fish that were vaccinated with the pLuc construct had mortalities varying from 85 to 100%. Fish vaccinated with the SVCVnc DNA vaccine had lower mortalities ranging from 10 to 50%. Differences in CPM among the pSGnc vaccine groups in the four trials were expected due to the varying challenge conditions, such as host age and challenge dose, as described in Table 3. The

Table 3
Challenge conditions in 4 trials of pSGnc DNA vaccinations in koi

Trial[a]	Koi weight (g)[b]	Koi age (months)[b]	Challenge dose (PFU/fish)[c]	Season (month/year)
A	1.5	4.0	5.0×10^4	February/2006
B	1.5	4.0	1.0×10^6	February/2006
C	3.5	10.5	1.0×10^5	April/2006
D	4.3	10–14.5	1.0×10^5	July/2006

[a] Rapid cold stressor was used in all 4 trials.
[b] Weight and age are at time of vaccination with a 10 μg dose of pSGnc DNA vaccine.
[c] Fish received an intraperitoneal injection of SVCVnc 28 days after vaccination.

Table 4
The results of four SVCV DNA vaccine trials conducted under various conditions defined in Table 3

Treatment group[a]	Trial A			Trial B			Trial C			Trial D		
	CPM (%)[b]	MDD[c]	RPS (%)[d]	CPM (%)[b]	MDD[c]	RPS (%)[d]	CPM (%)[b]	MDD[c]	RPS (%)[d]	CPM (%)[b]	MDD[c]	RPS[d]
NV	100	7.3	–	100	5.8	–	80	7.3	–	70	8.9	–
pLuc	100	8.1	0	100	8.1	0	85	9.4	−6	85	8.4	−21
pSGnc	50[e]	8.9	50	30[e]	6.7	70	10[e]	11.0	88	35[e]	8.7	50

[a] Non-vaccinated (NV) fish, pLuciferase (pLuc) vaccinated fish, and fish vaccinated with the SVCV DNA vaccine (pSGnc).
[b] Values listed as cumulative percent mortality (CPM) are average of duplicate treatment tanks.
[c] Mean day to death (MDD).
[d] Relative percent survival (RPS) calculations based on the non-vaccinated group as the comparative control.
[e] Indicates CPM values of pSGnc vaccinated fish were significantly different from pLuc vaccinated and non-vaccinated fish within each trial, with p-values of <0.05 by Mantel-Cox log-rank test (Systat 8.0).

Table 5
Virus titers in survivors after challenge in vaccine trials A–D

Trial	Non-vaccinated[a]	pLuciferase[a]	pSGnc[a]
A	No survivors	No survivors	1/6 (8.9×10^5)
B	No survivors	No survivors	2/6 (4.1×10^3)
C	3/3 (1.3×10^4)	2/2 (4.5×10^5)	0/10 (not detected)
D	6/6 (6.5×10^3)	3/3(1.8×10^4)	0/13 (not detected)

[a] Number of survivors positive for virus over the number of survivors tested for virus, followed in parentheses by the geometric mean (in pfu/g) of the positive survivor titers.

CPM, MDD, and RPS for each treatment group from the four trial experiments are summarized in Table 4. In each trial the pSGnc vaccinated fish mortality was significantly lower than the mortalities that occurred in either the non-vaccinated or pLuc-vaccinated fish. Thus, the pSGnc construct conferred significant protection in fish 28 days (546 D°) after vaccination, with RPS values ranging from 50 to 88%. The protection provided by the pSGnc DNA vaccine was significant at any of the three virus exposure levels tested ($p \leq 0.05$). In comparison, no protection (−21–0% RPS) was provided to the non-vaccinated (NV) fish groups or to the fish injected with the pLuc construct.

Virus was detected in 98% of the dead fish ($n = 96$) from the treatment groups exposed to virus from all four trials. The high virus concentrations (7.9×10^5 to 1.8×10^7 PFU/g) corroborated that fish demise was most likely due to SVCV infection. Among fish that survived the challenges the prevalence and virus concentration was lower than in fish that died, ranging from 4.1×10^3 to 4.5×10^5 PFU/g. The survivors tested from the NV and pLuc groups in every trial were all positive for virus. Survivors in the pSGnc vaccinated treatment groups had either low prevalence (3/12 fish in trials A and B) or no virus (0/23 fish in trials C and D) 30 days after virus exposure (Table 5).

4. Discussion

One of the primary goals of this work was the development of a challenge model that could reliably assess vaccine efficacy. The first fish tested as a possible cyprinid host were goldfish, since they were readily available, easy to rear, and reported to be susceptible to SVCV [11,41]. However, the goldfish we tested had little to no susceptibility to the SVCVnc, possibly due in part to their larger size (average 14.6 g). Koi, from a domestic US stock, were then tested since there is an established koi production and distribution industry in North America, and they were previously susceptible to at least three US strains of SVCV [11,14]. These specific pathogen-free koi have reliably been susceptible to both immersion and injection challenges with SVCVnc in our aquatic BSL-3 laboratory.

The initial challenge model protocol included a 2-week cold stress period prior to virus challenge to mimic the temperatures that typically occur before a SVCV outbreak. Many researchers have a suggested a temperature stressor is needed [1,4,42,43] to initiate successful SVCV infection in experimental models. Other researchers have suggested that a temperature stressor may not be the sole initiator of a successful SVCV infection in fish exposed to virus [3]. It is possible that any environmental stressor (handling, pollutants, etc.) acting alone or in conjunction with water temperature fluctuations may facilitate SVCV outbreaks. Our experiment demonstrated no significant difference in mortalities between koi challenged after being held for 2 weeks at colder water temperatures versus koi challenged immediately after transferring from stock water temperatures of ∼18 °C to BSL-3 laboratory water temperatures ranging from 10 to 12 °C. Our final challenge model included this rapid cold stressor on the day of virus challenge and utilized koi as the susceptible fish host species to SVCVnc. These initial experiments also indicated that koi would be a suitable target species for vaccine development.

The pSGnc vaccinated fish challenged 28 days (546 °D) after vaccination had significantly lower mortalities compared to the pLuc vaccinated fish and non-vaccinated control fish. The RPS values of 50–88% conveyed by the pSGnc vaccine in all four trials were comparable to or higher than RPS values reported for previously tested European genotype SVCV DNA constructs [28]. Since these were the first vaccination trials to test the pSGnc vaccine, the vaccine dose of 10 μg was higher than the standard doses (0.1–1.0 μg) used in IHNV and VHSV DNA vaccinations [27]. Despite using a higher vaccine dosage the pSGnc RPS values were lower than RPS values (>90%) typically reported for the novirhabdovirus (IHNV and VHSV) DNA vaccines [27]. Taxonomically SVCV is classified in the vesiculovirus genus and not the novirhadovirus genus. Thus structural and functional properties associated with fish vesiculoviruses may be a possible basis for the lower protection induced by the pSGnc vaccination. The absence of virus in the majority of the survivors vaccinated with pSGnc vaccine indicates that the fish had cleared the virus and completely recovered. The presence of virus in a few survivors from trials A and B may be attributed to the age of the fish used in those experiments and that the highest virus challenge dose was used in trial B. The younger koi (∼4 months old) used in the first trial studies (A and B) were at the fry life history stage and this may contribute to a slower clearance of the virus due to a less developed immune system.

The four trial studies demonstrated that our SVCV DNA vaccine consistently bestowed significant protection against a homologous North American SVCV isolate (SVCVnc) in immunized koi. Significant protection was demonstrated in the pSGnc-vaccinated fish tested under varying conditions (i.e., host age, virus dose, and season). Although it was not apparent which condition might be responsible for the variance observed in RPS, in every case the vaccine provided significant protection. Variation in the RPS obtained in the four trials did not correlate with factors such as fish size/age or challenge dose, but may have been impacted by vaccine leakage or unidentified differences between trial conditions. Future pSGnc studies will test the efficacy of the vaccine using lower inoculum doses and test the cross-protection against other North American and European strains of SVCV. The pSGnc construct is the first SVCV DNA vaccine to contain the G-gene from the Ia genogroup and the most efficacious vaccine reported yet for any SVCV DNA construct. This is also the first report of any SVCV DNA vaccine being tested successfully in koi. We anticipate this efficacious SVCV DNA vaccine can be utilized as a prophylactic tool to stem the invasion of this exotic virus in North America (U.S. provisional patent 60/959,928 filed 26 June 2007).

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References

[1] Fijan N, Petrinec Z, Sulimanovic D, Zwillenberg LO. Isolation of viral causative agent from the acute form of infectious dropsy of carp. Vet Archives 1971;41:125–38.

[2] Ahne W, Bjorklund HV, Essbauer S, Fijan N, Kurath G, Winton JR. Spring viremia of carp (SVC). Dis Aquat Organ 2002;52(3):261–72.
[3] Dixon P. Impacts, control and regulation of SVCV in wild and cultured fish in western Europe with a history of SVCV strains from China. In: Proceedings of the 30th Eastern fish health workshop, spring viremia of carp virus continuing education course. 2005.
[4] Ahne W. The influence of environmental temperature and infection route on the immune response of carp (*Cyprinus carpio*) to spring viremia of carp virus (SVCV). Vet Immunol Immunopathol 1986;12(1–4):383–6.
[5] Fijan N. Vaccination against spring viraemia of carp. Fish Vaccination 1988:204–15.
[6] Fijan N. Spring viremia of carp and other viral diseases of warm-water fish. In: Woo PTK, Bruno DW, editors. Fish diseases and disorders, vol. 3. Oxon: CAB International; 1999. p. 177–244.
[7] OIE. Office International des Epizooties Manual of diagnostic for Aquatic Animals, Spring Viremia of Carp. OIE; 2006. Chapter 2.1.4.
[8] Haenen OLM, Davidse A. Comparative pathogenicity of two strains of pike fry rhabdovirus and spring viremia of carp virus for young roach, common carp, grass carp and rainbow trout. Dis Aquat Organ 1993;15:87–92.
[9] Sanders GE, Batts WN, Winton JR. Susceptibility of zebrafish (*Danio rerio*) to a model pathogen, spring viremia of carp virus. Comp Med 2003;53:514–21.
[10] Svetlana J, Ivetic V, Radosavljevic V, Rhabdovirus. Carpio as a causative agent of disease in rainbow trout (*Oncorhynchus mykiss* – Walbaum). Acta Vet (Beograd) 2006;56(5–6):553–8.
[11] Goodwin AE. First report of spring viremia of carp virus (SVCV) in North America. J Aquat Anim Health 2002;14(3):161–4.
[12] United States Department of Agriculture (USDA) Animal Protection Health Inspection Service (APHIS) Impact Worksheet. July 17, 2002; 3 p.
[13] Dikkeboom AL, Radi C, Toohey-Kurth K. First report of spring viremia of carp virus (SVCV) in wild common carp in North America. J Aquat Anim Health 2004;16:169–78.
[14] Warg JV, Dikkeboom AL, Goodwin AE, Snekvik K, Whitney J. Comparison of multiple genes of spring viremia of carp viruses isolated in the United States. Virus Genes 2007;35:87–95.
[15] Garver KA, Dwilow AG, Richard J, Booth TF, Beniac DR, Souter BW. First detection and confirmation of spring viremia of carp virus (SVCV) in common carp, *Cyprinus carpio* (L) from Hamilton Harbour in Lake Ontario, Canada. J Fish Dis 2007;30:665–71.
[16] Fijan N, Matasin Z, Jeney Z, Olah J, Zwillenberg LO. Isolation of Rhabdovirus carpio from sheatfish (*Silurus glanis*) fry. Symp Biol Hung 1984;23:17–24.
[17] Wolf K. Infectious hematopoietic necrosis virus. In: Fish viruses and fish viral diseases. Ithaca, NY: Cornell University Press; 1988. p. 191–211.
[18] Tesarcik J, Macura B, Rehulka J. Evaluation of a broader clinical experiment with the application of a biopreparation for the control of the spring viraemia of carp in 1977. Bull VURH Vodnany 1978;14:3–6.
[19] Tesarcik J, Macura B. Field carp vaccination against spring viremia of the fish farms of the State Fishery. Bull VURH Vodnany 1981;17:3–11.
[20] Macura B, Tesarcik J, Rehulka J. Survey methods of specific immunoprophylaxis of carp spring viremia in Czechoslovakia. Pr VURH Vodnany 1983;12:50–6.
[21] Tesarcik J, Macura B, Rehulka J, Hrdonka M, Konasova V. Summarized results of pilot vaccination of carp against spring viremia in Czech Socialist Republic. Pr VURH Vodany 1984;13:68–74.
[22] Tesarcik J, Macura B. Spring viremia of carp—development of a vaccine in Czechoslovakia. In: Fijan N, Cvetnic S, Wikerhauser T, editors. Ichthyopathology in aquaculture. Zagreb: JAZU; 1988.
[23] Fijan N. Vaccination of fish in European pond culture: prospects and constraints. Symp Biol Hung 1984;23:233–41.
[24] Liu MA, Wahren B, Karlsson Hedestam GB. DNA vaccines: recent developments and future possibilities. Hum Gene Ther 2006;17:1051–61.
[25] Kurath G. Overview of recent DNA vaccine development for fish. In: Midlyng PJ, editor. Progress in fish vaccinology. Basel: Karger; 2005. p. 201–13.
[26] Jechlinger W. Optimization and delivery of plasmid DNA for vaccination. Exp Rev Vaccines 2006;5(6):803–25.
[27] Kurath G, Purcell MK, Garver KA. Fish rhabdovirus models for understanding host response to DNA vaccines. CAB Rev Perspect Agric Vet Sci Nutr Nat Resour 2007;2:No. 048.
[28] Kanellos T, Sylvester ID, D'Mello F, Howard CR, Mackie A, Dixon PF, et al. DNA vaccination can protect *Cyprinus Carpio* against spring viraemia of carp virus. Vaccine 2006;24(23):4927–33.
[29] Fauquet CM, Maniloff J, Desselberger U, Ball LA, editors. Virus taxonomy—eighth report of the international committee on the taxonomy of viruses. New York: Elsevier Academic Press; 2005.
[30] Bjorklund HV, Emmenegger EJ, Kurath G. Comparison of the polymerases (L genes) of spring viremia of carp virus and infectious hematopoietic necrosis virus. Vet Res 1995;26:394–8.
[31] Corbeil S, LaPatra SE, Anderson ED, Kurath G. Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous virus strains of infectious hematopoietic necrosis virus. Vaccine 2000;18:2817–24.
[32] Lorenzen N, LaPatra SE. DNA vaccines for aquacultured fish. Rev Sci Tech OIE 2005;24:201–13.
[33] Fijan N, Sulimanovic D, Bearzotti M, Muzinic D, Zwillenberg LO, Chilmonczyk S, et al. Some properties of the *epithelima papulosum cyprinid* (EPC) cell line from carp *Cyprinus carpio*. Ann Instit Pasteur Virol 1983;134E:207–20.
[34] Batts WN, Winton JR. Enhanced detection of infectious hematopoietic necrosis virus and other viruses by pretreatment of cell monolayers with polyethylene glycol. J Aquat Anim Health 1989;1:284–90.
[35] Emmenegger EJ, Meyers TR, Burton TO, Kurath G. Genetic diversity and epidemiology of infectious hematopoietic necrosis virus in Alaska. Dis Aquat Organ 2000;40(3):163–76.
[36] Bjorklund HV, Higman KH, Kurath G. The glycoprotein genes and gene junctions of the fish rhabdoviruses spring viremia of carp and hirame rhabdovirus: analysis of relationships with other rhabdoviruses. Virus Res 1996;42:65–80.
[37] Emmenegger EJ, Troyer RM, Kurath G. Characterization of the mutant spectra of a fish RNA virus within individual hosts during natural infections. Virus Res 2003;(96):15–25.
[38] Corbeil S, LaPatra SE, Anderson ED, Jones J, Vincent B, Hsu YL, et al. Evaluation of the protective immunogenicity of the N, P, M, NV and G proteins of infectious hematopoietic necrosis virus in rainbow trout *Oncorhynchus mykiss* using DNA vaccines. Dis Aquat Organ 1999;39(1):29–36.
[39] Saporito-Irwin SM, Geist RT, Gutmann DH. Ammonium acetate protocol for the preparation of plasmid DNA suitable for mammalian transfections. Biotechniques 1997;23:424–7.
[40] Johnson KA, Flynn JK, Amend DF. Onset of immunity in salmonid fry vaccinated by direct immersion in *Vibrio anguillarum* and *Yersinia ruckeri* bacterins. J Fish Dis 1982;5:197–205.
[41] Horvath L, Tamas G, Seagrave C. Carp and pond fish culture. New York, NY: Blackwell Publishing; 2002, 192 p.
[42] Ahne W. Rhabdovirus carpio-Infektion beim Karpfen (*Cyprinus carpio*): Untersucheungen uber Reaktionene des Wirtsorganismus. Fortschr Veterinarmed 1980;30:180–3.
[43] Baudouy AM, Danton M, Merle G. Experimental infection of susceptible carp fingerlings with spring viremia of carp virus under wintering environmental conditions. In: Ahne W, editor. Fish diseases. Third COPRAQ session. Berlin: Springer-Verlag; 1980. p. 23–7.
[44] Stone DM, Ahne W, Denham KL, Dixon PF, Liu CT-Y, Sheppard AM, et al. Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhabdovirus isolates reveals four genogroups. Dis Aquat Organ 2003;53(3):203–10.