Spring Viremia of Carp in the United States of America: Evaluation of Current Diagnostics

RAGHUNATH SHIVAPPA[1*], SHANNON KOZLOWICZ[1], JILL ROLLAND[2], FLAVIO CORSIN[3], KEITH WAY[4] and JAY LEVINE[1]

[1]Department of Population Health and Pathobiology, College of Veterinary Medicine North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606, USA

[2] United States Department of Agriculture/Animal and Plant Health Inspection Service, 4700 River Road, Unit 46, Riverdale, MD 20737, USA

[3] Network of Aquaculture Centers in Asia-Pacific PO Box 1040, Kasetsart Post Office, Bangkok, 10903, Thailand

[4]Center for Environment, Fisheries and Aquaculture Science Weymouth Laboratory, Barrack Road, The Nothe, Weymouth DT4 8UB UK

**Current Address: Diosynth Biotechnology, A part of Schering-Plough Corporation 3000 Weston Parkway, Cary, NC 27513 USA*

ABSTRACT

Accurate diagnosis is essential for prevention and avoiding the spread of foreign animal diseases. Spring viremia of carp (SVC), which was considered an exotic disease in the US, was first detected in April 2002. The United States Department of Agriculture (USDA) responded immediately to prevent the spread of the disease. Depopulation of all infected and exposed fish followed by quarantine and a surveillance program were initiated in each facility. There are several diagnostic tests that are available for the detection of SVC virus (SVCV); however, the tests have not been validated. *in vitro* sensitivity and specificity for several SVCV diagnostic tests were evaluated. Serial dilutions of SVCV virus as low as 10^1 $TCID_{50}$/mL were used to evaluate the *in vitro* sensitivity of SVCV diagnostic tests. *In vitro* specificity was evaluated with serial dilutions of five different strains of

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Corresponding author: Raghunath Shivappa, raghunath_shivappa@ncsu.edu

fish rhabdoviruses using the SVCV diagnostic tests. The study showed that a minimum viral titer of 10^5 $TCID_{50}$/mL is required to detect the virus in cell culture within 48 hours. Molecular and serological based techniques proved to be more sensitive and were able to detect the virus in less time than virus culture. Among these tests, reverse transcriptase-polymerase chain reaction (RT-PCR) was the most sensitive and detected the virus at 10^1 $TCID_{50}$/mL with specific primers designed for the US isolate. With regards to specificity, RT-PCR and enzyme linked immunosorbent assay (ELISA) assays did not detect any fish rhabdovirus other than SVCV. Although *in vivo* studies are pending, the preliminary findings of this study indicate that RT-PCR could be recommended as a test of choice for rapid and accurate diagnosis of a US SVCV isolate.

INTRODUCTION

Outbreaks of spring viremia in carp have been recognized by European carp farmers for centuries. The disease is associated with a rhabdovirus, spring viremia of carp virus (SVCV) or *Rhabdovirus carpio*, which is readily transmitted in the feces of infected fish. The virus is environmentally stable and is possibly transferred between surface waters and aquaculture farms by waterfowl, fish parasites, and fomites (Fijan *et al.,* 1971; Ahne *et al.,* 2002). External clinical signs of the disease include an extended abdomen, exophthalmia and an inflamed vent. Internally, the fish exhibit ascites and petechial hemorrhages of the internal wall of the swimbladder. The disease is generally associated with high mortality; often approaching 70% in young fish (Ahne *et al.,* 2002). Consequently, introduction of the virus into a commercial hatchery or aquaculture facility can have profound economic effects.

In April 2002, SVC was reported for the first time in the US in one of the largest koi farms in the United States of America (US) with operations consisting of approximately 202 ponds spanning the states of North Carolina (NC) and Virginia (VA). The World Organisation for Animal Health (OIE) confirmed the presence of SVCV in the affected fish. As SVC is considered a foreign animal disease, State quarantines were placed on the facilities upon confirmation of the disease. A joint request from States and industry was made to the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) for assistance with depopulation, cleaning, disinfection and indemnity. In March of 2003, APHIS received approval to implement an SVC control and indemnity program using emergency funding. A depopulation, cleaning and disinfection effort of this magnitude had not previously been undertaken at a fish farm. The logistics of carrying out the eradication effort required input from several State and Federal agencies as well as input from SVC experts both within and outside of the United States. Depopulation efforts commenced in early July of 2003 and the State quarantines on the facility were released by the end of October 2003 following completion of cleaning and disinfection. In addition to efforts put forth at the infected premise, APHIS instituted a nationwide voluntary surveillance. No positive cases have been identified through the surveillance program. Following the NC/VA outbreak, episodes of mass mortalities of wild common carp were reported in Wisconsin in the spring of 2002 (Goodwin *et al*., 2002, Marcquenski

et al., 2003, Dikkeboom *et al*., 2004). In July of 2004, SVC was also reported in a backyard pond in Washington State and in August of 2004, a commercial farm in Missouri was also diagnosed with SVC. APHIS depopulated, cleaned and disinfected these premises and also conducted epidemiologic investigations to try and determine the source of infection.

Since 2002, the presence of SVCV in the US has been extensively investigated in both wild and farmed cyprinid populations. A variety of serology and PCR-based assays have been developed to assist veterinarians and fish health professionals document the presence of SVCV. The OIE recommended diagnostic tests for SVCV include virus culture, reverse transcriptase-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody technique (IFAT). However, detection of SVCV by PCR or serologic detection of SVCV antibody must be confirmed by virus culture. Each of these tests has its own merits and deficiencies, varying in cost, labor and training requirements, sensitivity, specificity, and diagnostic predictive value. In this study, we measured and compared the *in vitro* accuracy of currently available diagnostic techniques for SVCV. Our aim in this study was to identify the most appropriate currently available diagnostic techniques for detection of SVCV and where appropriate, refine them and enhance their accuracy.

MATERIALS AND METHODS

Virus Culture

Epithelioma papulosum cyprini (EPC) cells were maintained at 20°C in Leibovitz-15 medium supplemented with 10% fetal bovine serum (FBS). An isolate of SVCV from the NC/VA outbreak (referred to as the US isolate hereafter) was obtained from the Center for Environment, Fisheries, and Aquaculture Science (CEFAS), Weymouth, England (OIE Reference Laboratory for SVC). The viral supernatant was filtered using a 0.2-micrometer filter and propagated in EPC cells and purified. Briefly, EPC cells were infected with SVCV and after cytopathic effect (CPE) was observed, the cells were scraped into the medium and the crude virus was clarified by centrifuging at 5000 × *g* for 30 min at 4°C. The supernatant was aspirated and loaded carefully on a 26% sucrose cushion and subjected to ultracentrifugation at 120 000 *g* for 2 hr at 4°C. Finally, the virus pellet was resuspended in 500 µl of HO buffer (0.01 M Tris-Hcl, 0.25 M Nacl, 0.01M β-mecaptoehatnol), titrated by the method of Reed and Muench (1938) by 50% tissue culture infective dose ($TCID_{50}$) endpoint analyses and stored at –20°C until use. The titrated stock virus was serially diluted to yield 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 $TCID_{50}$/mL to perform the sensitivity assays.

To test the specificity of the assays, five fish rhabdoviruses including American and European strains of infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and pike fry rhabdovirus (PFRV) were used (provided by Dr. G. Kurath, USGS). The viruses were grown in EPC cells cultured in L-15 media at 15°C, purified and titrated as noted above. Viruses were serially diluted as noted above, but based on the results of the sensitivity studies only 10^3, 10^4, 10^5 and 10^6 $TCID_{50}$/mL were used for the *in vitro* specificity studies

Sensitivity of Virus Culture

Cells (EPC) grown in 25cm^2 flasks were infected in triplicate with 100 µl of serial dilutions of SVCV and incubated at 20^{o}C. The flasks were monitored for cytopathic effect every 24 hrs (OIE, 2003).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Viral RNA was extracted from undiluted and serially diluted viral supernatants from infected and uninfected EPC cells (negative control) using QIAamp viral RNA kit (Qiagen, MD) following the manufacturer's protocol. The extracted viral RNA and negative control RNA were subjected to one step RT-PCR reaction (Qiagen). Two sets of primers were designed based on the OIE manual (2003) and Stone *et al*., (2003) to amplify the 714 bp fragment of the SVCV glycoprotein (G) (Table 1). A degenerate primer set and a primer set specific for the US isolate were designed. The primer set specific for US isolate was designed based on the nucleotide sequence of the G protein (Gene bank accession number: AY527273). Using viral RNA as a template, RT was performed at 50°C for 30 min followed by 95°C for 15 min. The cDNA was then amplified by 30 and 40 cycles of 95°C for 1 min, 55 °C for 1min, 72 °C for 1 min, and a final extension step of 72°C for 10 min. The RT-PCR products were visualized after electrophoresis on a 1% agarose gel stained with SYBR green.

Table 1. Oligonucleotides used for testing the sensitivity and specificity of the RT-PCR diagnostic test for SVCV. Orientation of virus-specific sequence of the primer is shown as sense (+) or antisense (-).

Nucleotide Sequence	Orientation	Designation	Expected size (bp)
SVCV			
Degenerate Primer			
TCTTGGAGCCAAATAGCTCAGGTC	+	SVCVF1	718
AGATGGTATGGACCCCAATACATCACGCAC	-	SVCVR2	
US isolate specific			
AGATGGTACGGACCCCAATATATAACCC	+	SVCVNCF	718
TCTTGGGGCCAAATAACTCAAATCC	-	SVCVNCR	
IHNV			
TCAAGGGGGGAGTCCTCGA	+	IHNVF	786
CACCGTACTTTGCTGCTAC	-	IHNVR	
VHSV			
GGGGACCCCAGACTGT	+	VHSVF	811
TCTCTGTCACCTTGATCC	-	VHSVR	
Semi-nested Primer for SVCV and PFRV			
CTGGGGTTTCCACCTACCAGTTGC	+	PFRnestF	606

For specificity assays, viral RNA from undiluted and serially diluted fish rhabdoviruses (mentioned above) and the negative control were extracted and subjected to RT-PCR following the above protocol. Primers specific for IHNV and VHSV were designed based on the OIE manual and SVCV primers were employed for PFRV (Stone *et al*., 2003). Semi-nested RT-PCR based on Stone *et al*. (2003) was also performed on SVCV and PFRV to detect any cross reactivity that is believed to exist between these two viruses. The SVCV reverse primer from the above RT-PCR assay was used along with the forward primer that was designed based on Stone *et al.* (2003). The template from the previous RT-PCR reaction was then used to perform the semi-nested PCR assay with similar amplification conditions described above.

Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA assay was performed following the protocol of the CEFAS laboratory. Briefly, 96 well plates (in triplicate) were coated with 100 µl of anti SVCV γ-globulin at a concentration of 5 µg/mL in carbonate buffer and incubated overnight at 20°C. Following incubation, the plates were washed thrice with a buffer containing phosphate buffered saline and thiomersal (PBS-Thio). The plates were then blocked with 5% fish skin gelatin (FSG) at 37°C for 45 min. Using PBS+0.05%Tween 20 (PBST) buffer the plates were washed thrice. The plates were than loaded with 100 µl of undiluted and serially diluted SVCV viral samples (US isolate) along with a negative control (uninfected EPC cell supernatant) and incubated at 37°C for 30 min. Viral dilutions were made using the extraction buffer (PBST+Nonidet P-40). The plates were then rinsed with PBST followed by 3 washes. One hundred microlitres of a biotinylated anti SVCV IgG at a dilution of 1:1000 in PBST/FSG was then added to each well on the plate and incubated at 37°C for 30 min. The plates were washed as in the previous step and then 100 µl of ExtrAvidin horse radish peroxidase (HRPO) conjugate (Sigma MO, USA) diluted to 1:1000 in PBST/FSG was added to each well followed by incubation at 37°C for 30 min. Following washing as in the previous step, the plates were drained thoroughly by tapping the plates on absorbent paper. The substrate buffer (phosphate-citrate buffer with sodium perborate (Sigma) was made up according to the manufacturer's protocol and one tablet of tetramethylbenzidine dihydrochloride (TMB, Sigma) was added. This solution (100 µl) was then added to each sample well, plus two additional wells (blanks) and incubated at room temperature for 10 minutes. Once the positive controls showed a strong blue color the reaction was stopped using 25 µl of 10% H_2SO_4. The color was read in a microplate reader (ELX-800, Bio-Tek Instruments, VT, USA) at 450 nm (standard protocol from the CEFAS, laboratory). The mean optical density (OD) [absorbance value (A450)] after subtracting the blank value was recorded for each viral sample as well as negative controls using the KcJunior software (Bio-Tek Instruments).

Undiluted and diluted fish rhabdoviruses (mentioned above) and a negative control of either no EPC cells or EPC cells were subjected to ELISA under similar conditions as described above to determine the specificity of the assay. The assay with no EPC cells as a negative control used serial dilutions of all viruses while the one with EPC cells used only undiluted virus of all fish rhabdoviruses.

Indirect Fluorescent Antibody Technique (IFAT)

The IFAT test was conducted in 24-well plates following the protocol from the CEFAS laboratory. Fresh monolayers of EPC cells (24 hr old) were passaged into 24 well plates (in triplicate) and incubated for 3 hours at 20°C. Following incubation, cells were infected with undiluted and serially diluted viral supernatants of SVCV (US isolate) and incubated overnight at 20°C. Uninfected wells served as negative controls. One mL of PBS was then carefully added to each well and incubated at room temperature for 1 min. The buffer (PBS) was then carefully removed using a pipette and 0.5 mL of freshly made 80% aqueous acetone was added to each well and incubated at room temperature for 20 min. The acetone was removed and the plates were air-dried. For staining the plates, the anti SVCV γ-globulin, at a dilution of 1:300 in PBST, was added (200 µl) following addition and immediate removal of 0.5 mL of PBST to the air-dried wells. Following a 30 min incubation at 37°C, the antibody was removed and washed by adding 1 mL of PBST to each well and incubated at room temperature on an orbital shaker at 250 rpm for 10 min. The wash liquid was poured out and the wash was repeated once. The plate was tapped gently to remove excess fluid from the plate and 200 µl of 1:1000 dilution of goat anti-rabbit FITC conjugate (Sigma) was added to each well and incubated at 37°C for 30 min. The conjugate was removed after incubation and washed twice as in the previous step. After the final wash was removed, 200 µl of distilled water was added to each well and read immediately on an inverted fluorescent microscope.

For the specificity experiment, the test was carried out as described above with undiluted and serially diluted fish rhabdoviruses (as mentioned above).

Data Analysis

In-vitro sensitivity of each test was determined as the lowest concentration detectable by each test while *in-vitro* specificity was recorded as a categorical (dichotomous) variable, either detection or no detection of other fish rhabdoviruses for each test. For ELISA, the absorbance values shown are the mean of three plates with two wells per plate for each sample. Descriptive statistics were calculated using Microsoft Excel (Microsoft Corporation, Seattle, Washington). Minitab (Minitab Inc., State College, Pennsylvania) was used to conduct a one-sided Mann-Whitney-U test to compare assay results with the respective control (Armitage and Berry, 2002). A p-value $< .05$ was considered statistically significant.

RESULTS

Virus Culture

Monolayers of EPC cells in 25cm^2 were infected with different dilutions of SVCV and monitored every 24 hrs for the appearance of CPE. Cells showed extensive CPE within 48 hrs in the undiluted (10^6 $TCID_{50}$/mL) and 10^5 $TCID_{50}$/mL SVCV infected flasks. CPE

progressed in the other dilutions and complete CPE could be noticed only after 7 days post infection in the lowest dilution (10^1TCID$_{50}$/mL). Negative control cells (uninfected EPC cells) did not show signs of CPE.

RT-PCR

Viral RNA extracted from several dilutions of SVCV was subjected to RT-PCR following the OIE protocol with two sets of primers. A specific product of 714 bp was recorded with both the primers. With the degenerate primer set, SVCV could be detected up to 10^5 TCID$_{50}$/mL with 30 cycles of cDNA amplification (data not shown). Upon increasing the amplification cycles to 40 cycles, SVCV could be detected even at 10^4 TCID$_{50}$/mL (Figure 1). A tremendous increase in the capability of RT-PCR to detect SVCV was recorded when the primer set designed based on the nucleotide sequence of the G protein of the US isolate was used. The virus could be detected even at the lowest dilution of 10^1 TCID$_{50}$/mL while the negative control (RNA extracted from uninfected EPC cells) was not amplified (Figure 2).

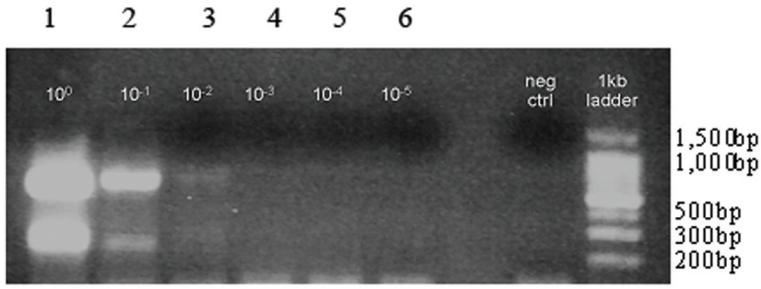

Figure 1. *In vitro* sensitivity analysis of the SVCV RT-PCR diagnostic test with degenerate primers. RT-PCR was performed on serial dilutions of SVCV ranging between 10^6 TCID$_{50}$/mL to 10^1 TCID$_{50}$/mL (lanes 1-6).

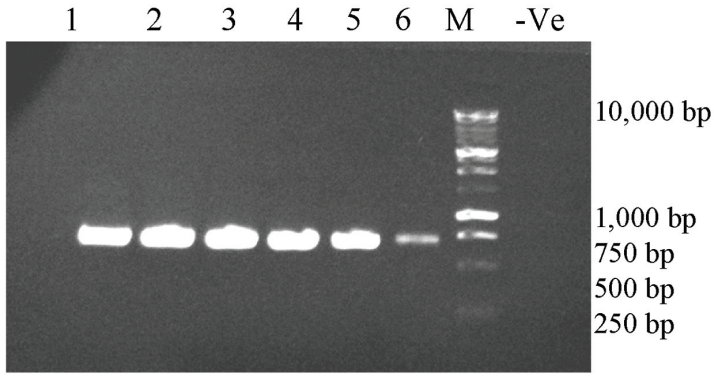

Figure 2. *In vitro* sensitivity analysis of SVCV RT-PCR diagnostic test with US isolate specific primers. Serial dilutions of SVCV ranging between 10^6 TCID$_{50}$/mL to 10^1 TCID$_{50}$/mL (lanes 1-6) were made and subjected to RT-PCR.

Several fish rhabdoviruses were tested concurrently with SVCV virus to compare the specificity of the assay. It was found that both primer sets could detect only the US isolate of SVCV (Figure 3). However, when virus specific primers for IHNV, VHSV and PFRV were employed, RT-PCR products specific for IHNV (786 bp product) and VHSV (811 bp) were amplified (Figure 4). The SVCV primers based on Stone et al., (2003), which were used for PFRV, did not yield any product (Figure 4). A semi-nested RT-PCR was also conducted with SVCV and PFRV using the US isolate specific reverse primer and a new forward primer (PFRnesF). However, no products could be generated for either of the viruses (Figure 4).

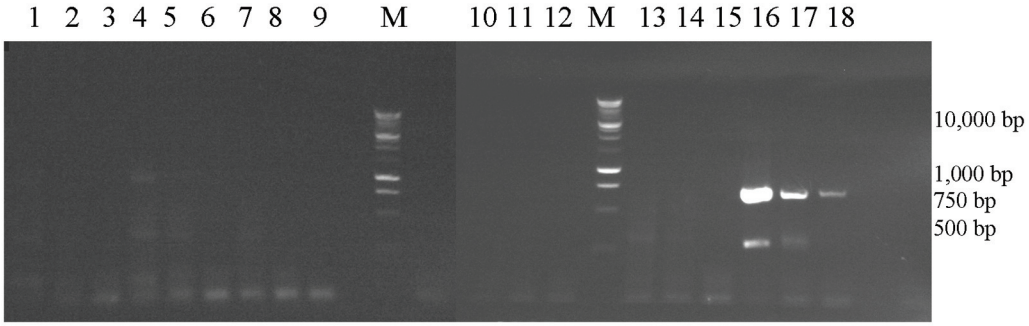

Figure 3. *In vitro* specificity analyses of SVCV RT-PCR assay using different strains of fish rhabdoviruses. A two fold serial dilution of 5 strains of fish rhabdoviruses and SVCV primers based on the OIE manual were used. Lanes 1-3: IHNV (European strain), 4-6: IHNV (American strain), 7-9: VHSV (European strain), 10-12: VHSV (American strain), 13-15: PFRV, 16-18: SVCV.

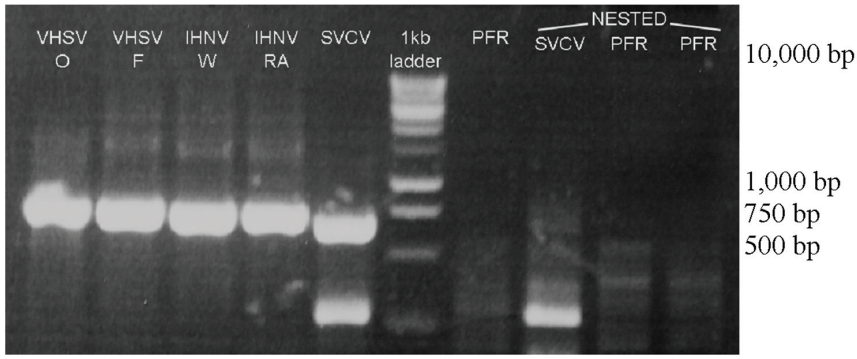

Figure 4. Virus specific RT-PCR for different fish rhabdoviruses using primers specific to IHNV, VHSV and SVCV. The primers used for PFRV were the same as for SVCV. Semi-nested RT-PCR was employed to test for cross reactivity. No nested-RT PCR products could be generated.

ELISA

Serially diluted SVCV (US isolate) was tested in an ELISA based on a CEFAS laboratory protocol. It was found that there was a significant difference ($p<.05$) in absorbance at 10^6

$TCID_{50}$/mL (undiluted), 10^5 $TCID_{50}$/mL, and 10^4 $TCID_{50}$/mL dilutions compared to the negative control (Figure 5). No significant absorbance values could be recorded in the lower dilutions.

Specificity assays for ELISA showed a significant difference ($p<.05$) in absorbance of all viruses at the undiluted concentration compared to the negative control without EPC cells (Figure 6). In this experiment, a significant difference in absorbance for SVCV was only

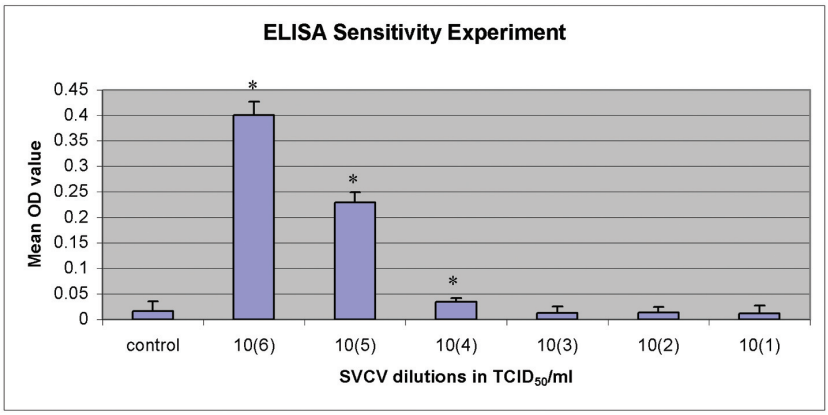

Figure 5. *In vitro* sensitivity analysis of the SVCV ELISA diagnostic test. ELISA was performed on serially diluted SVCV using anti-serum raised against the virus. The OD was read at 450nm and the values shown are the mean values for three plates with two wells each per plate. Significance at the 0.05 level using a one-sided Mann-Whitney test is indicated by *.

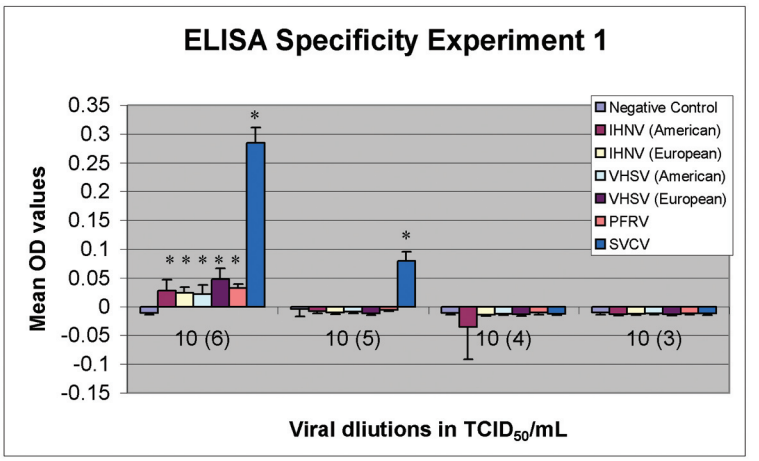

Figure 6. *In vitro* specificity analysis of SVCV ELISA diagnostic test. Serially diluted SVCV, IHNV, VHSV and PFRV were subjected to ELISA using anti-serum raised against the virus. The OD was read at 450nm and the values shown are the mean values from three plates with two wells each per plate. Negative control did not contain EPC cells. Significance at the 0.05 level using a one-sided Mann-Whitney test is indicated by *.

seen at 10^6 $TCID_{50}$/mL (undiluted) and 10^5 $TCID_{50}$/mL. In another experiment, when EPC cells were included as the negative control, only the absorbance of the undiluted US isolate of SVCV differed significantly ($p<.05$) from other fish rhabdoviruses and the negative control (Figure 7).

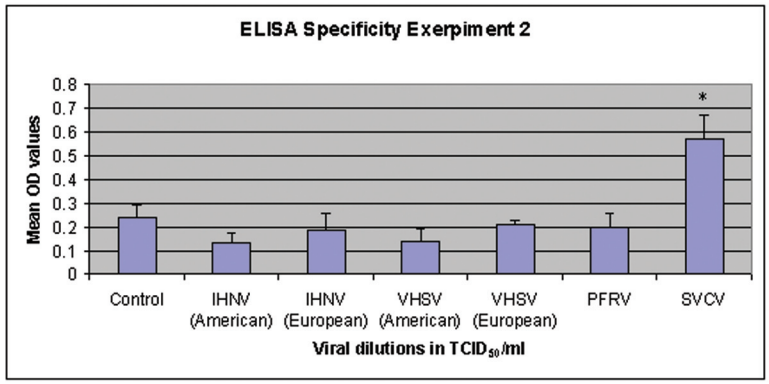

Figure 7. *In vitro* specificity analysis of SVCV ELISA diagnostic test. Undiluted SVCV, IHNV, VHSV and PFRV were subjected to ELISA using anti-serum raised against the virus. The OD was read at 450nm and the values shown are the mean values from three plates with two wells each per plate. Negative control contained EPC cells. Significance at the 0.05 level using a one-sided Mann-Whitney test is indicated by *.

IFAT

The ability of IFAT to detect SVCV at different dilutions of SVCV (US isolate) was carried out following the CEFAS laboratory protocol. The test showed detection of the virus at 10^6 and 10^5 $TCID_{50}$/mL dilutions. No detectable fluorescence could be detected in lower dilutions. The test was repeated three times and consistent results were obtained.

The specificity assay for IFAT using different fish rhabdoviruses were not conclusive. Although we were able to detect only SVCV in the first experiment, we were not able to reproduce the results and cross reaction with other rhabdoviruses was recorded in subsequent experiments (data not shown).

DISCUSSION

Rapid detection of a pathogen is an essential component of all surveillance programs aimed at preventing the introduction of a foreign animal (transboundary) disease. The more rapidly the presence of the pathogen is identified, the more rapidly infected populations can be treated or depopulated. Spring viremia of carp virus is a serious pathogen of carp, which was recently introduced into the United States. (Goodwin, 2002). Rapid and accurate detection of the virus is vital to efforts to prevent the further introductions and spread of the virus. In addition to virus culture, several diagnostic tests have been developed to detect SVCV. However, OIE recommends that the detection of virus by any of the serological

or PCR based methods be confirmed by virus culture. Based on our studies, virus culture, although very sensitive, can take up to 7 days for detection of very low virus levels. Consequently, it does not meet the standard of necessity for rapid detection. A seven-day delay could have serious economic consequences for a producer that waits to take control measures to prevent further mortality. We compared virus culture with other alternative assays available to detect SVCV. Of the assays we evaluated, RT-PCR appears to offer great promise for the rapid accurate detection of SVCV. The US isolate from NC/VA outbreak was detected even at very low levels (10^1 $TCID_{50}$/mL), comparable to virus concentrations detectable by virus culture. However, the time required to conduct RT-PCR analysis of a sample is markedly less than the time required to obtain culture results. Similar sensitivity was reported by Koutna *et al*. (2003). They tested a combination of RT-PCR and nested PCR for SVCV on several clinical samples and reported a sensitivity of 10^{-1} $TCID_{50}$/mL. Our results for the US isolate showed similar sensitivity and hence can be recommended as a method of choice to confirm the presence of US isolates of SVCV. Results also showed that the sensitivity of OIE recommended RT-PCR for SVCV can be increased by one log by increasing the amplification cycles to 40 instead of 30. Also, the fact that the NC specific primer was more sensitive compared to the degenerate primer suggests that the US isolate from NC is different from the reference strain. This observation is in agreement with the recent study by Dikkeboom *et al.* (2004) who reported that the US isolates of NC and Cedar Lakes, WI are more closely related to an Asian strain than the European reference strain. A follow up test of this primer for its applicability to all US isolates would be useful. Efforts are in progress in our laboratory to procure all the isolates needed to conduct these tests.

Specificity was excellent for *in vitro* RT-PCR, as neither the RT-PCR nor the nested PCR detected any fish rhabdovirus other than SVCV (Figure 4). Although, it is reported that the PCR is non-specific and detects PFRV-like viruses in most cases (Stone *et al*., 2003), no such cross reactivity was seen in this study. However, according to Stone (personal communication) the semi-nested RT PCR product can be seen after 30 PCR cycles but disappears upon increasing the number of cycles. In our study, we used 40 PCR cycles in all assays, which could be one of the reasons we did not detect any semi-nested PCR products. This suggestion will be incorporated into follow up tests and *in vivo* studies. Although our RT-PCR assay shows high *In vitro* sensitivity and specificity and no cross reactivity with PFRV, it is premature to believe that our primer is highly specific for SVCV before *in vivo* studies are performed.

The serological based methods like ELISA and IFAT could also detect SVCV. However, the sensitivity was low compared to RT-PCR. Although these tests are routinely practiced to confirm the presence of SVCV and are recommended by OIE, they are time consuming, cumbersome, and often difficult to replicate. Additional studies aimed at optimizing these techniques are needed to overcome the drawbacks associated with these tests.

In summary, the results from our study clearly indicate that RT-PCR is fast, accurate, reproducible, and could definitely be recommended as a method of choice for diagnosing SVCV. The method can be completed in less than 24 hrs as compared to other serological based techniques or virus culture. Thus RT-PCR provides significant saving in time, cost and materials. These observations need to be further validated by conducting studies using

naturally or artificially infected fish. Virus culture is currently required by OIE guidelines to confirm the results of PCR-based assays because of concerns about detecting nonviable viral RNA. The RT-PCR assay could be used as a screening tool to identify SVCV, which could then initiate response activities such as quarantine and strict biosecurity protocols. Due to the possibility of RT-PCR detecting nonviable virus, all depopulation events can be deferred until the results of virus isolation is confirmed. Uniform OIE approved test guidelines and test conditions would enhance confidence in test RT-PCR results, and with this standardization the results of this assay could be considered definitive indication that the virus is present. Protocol standardization and a standard procedure for quality assurance assessment of individual laboratories should be developed to ensure diagnostic consistency between laboratories.

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