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Analytical validation of two RT-qPCR tests and detection of spring viremia of carp virus (SVCV) in persistently infected koi *Cyprinus carpio*

Sharon C. Clouthier[1,*], Tamara Schroeder[1], Emma K. Bueren[2,4], Eric D. Anderson[3], Eveline Emmenegger[2]

[1]Fisheries & Oceans Canada, Freshwater Institute, Winnipeg, Manitoba R3T 2N6, Canada
[2]US Geological Survey, Western Fisheries Research Center, Seattle, WA 98115, USA
[3]Box 28, Group 30, RR2, Ste Anne, Manitoba R5H 1R2, Canada

[4]*Present address:* Department of Biological Sciences, Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA 24061, USA

ABSTRACT: Spring viremia of carp virus (SVCV) ia a carp sprivivirus and a member of the genus *Sprivivirus* within the family *Rhabdoviridae*. The virus is the etiological agent of spring viremia of carp, a disease of cyprinid species including koi *Cyprinus carpio* L. and notifiable to the World Organisation for Animal Health. The goal of this study was to explore hypotheses regarding intergenogroup (Ia to Id) SVCV infection dynamics in juvenile koi and contemporaneously create new reverse-transcription quantitative PCR (RT-qPCR) assays and validate their analytical sensitivity, specificity (ASp) and repeatability for diagnostic detection of SVCV. RT-qPCR diagnostic tests targeting the SVCV nucleoprotein (Q2N) or glycoprotein (Q1G) nucleotides were pan-specific for isolates typed to SVCV genogroups Ia to Id. The Q2N test had broader ASp than Q1G because Q1G did not detect SVCV isolate 20120450 and Q2N displayed occasional detection of pike fry sprivivirus isolate V76. Neither test cross-reacted with other rhabdoviruses, infectious pancreatic necrosis virus or co-localizing cyprinid herpesvirus 3. Both tests were sensitive with observed 50% limits of detection of 3 plasmid copies and high repeatability. Test analysis of koi immersed in SVCV showed that the virus could be detected for at least 167 d following exposure and that titer, prevalence, replicative rate and persistence in koi were correlated significantly with virus virulence. In this context, high virulence SVCV isolates were more prevalent, reached higher titers quicker and persisted in koi for longer periods of time relative to moderate and low virulence isolates.

KEY WORDS: SVCV · RT-qPCR · Analytical specificity · Analytical sensitivity · Persistent infection · Koi · *Sprivivirus*

1. INTRODUCTION

Spring viremia of carp virus (SVCV) belongs to the genus *Sprivivirus* within the family *Rhabdoviridae* in the order *Mononegavirales* (ICTV 2019). The disease spring viremia of carp (SVC) is notifiable to the World Organisation for Animal Health (OIE) (OIE 2019a) and reportable to the Canadian Food Inspection Agency (CFIA 2019). The primary host for SVCV worldwide is common carp *Cyprinus carpio* L. (Fijan 1999), but natural outbreaks of the disease have occurred in other cyprinid fish species (Ahne et al. 2002). The host range might be broader than currently recognized, since cell lines from birds, reptiles

*Corresponding author: sharon.clouthier@dfo-mpo.gc.ca

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and mammals support growth of SVCV (Bachmann & Ahne 1974, Clark & Soriano 1974). The discovery of SVCV in Chinese firebelly newts (Ip et al. 2016) extends the host range of the virus from fish to amphibians.

SVCV can be virulent in juvenile carp (70–100% mortality) during their first year, and die offs are observed in the spring when the water temperature is between 10 and 17°C (Ahne et al. 2002). Mortality in older, mature fish is not common but can occur in populations that have never been exposed to the virus (Ahne et al. 2002, Dikkeboom et al. 2004). The dynamic of SVC is dependent on a number of factors including virus isolate, water temperature, host age and condition, population density and stress factors (Ahne et al. 2002). The gill is considered the primary portal of entry into the host, with subsequent viremia and dissemination of the endotheliotropic virus to the liver, kidney, spleen, heart and gastrointestinal tract (Ahne 1977, 1978). Like other fish rhabdoviruses, SVCV is also neurotropic (Wang et al. 2017), a feature that may allow the virus to exploit other entry routes into the host and establish a persistent type of infection in the brain (Sepahi et al. 2019). Virus is initially observed in kidney tissue followed by higher titers in liver and spleen tissue with concomitant decline in kidney titers (Faisal & Ahne 1984). Little is known regarding the kinetics and titer of SVCV in brain tissue except that the titer is typically lower compared to kidney tissue (Wolf 1988).

Some strains of SVCV can persist in carp for more than 70 d (Ahne et al. 2002), but few (if any) studies have investigated inter-genogroup differences in persistence, a gap identified by the OIE SVCV reference laboratory (OIE 2019b). Horizontal transmission of the virus can occur from asymptomatic apparently healthy adult survivors (Bekesi & Csontos 1985) that shed virus into the water through their excreta (Wolf 1988, Ahne et al. 2002) or via their reproductive products (Bekesi & Csontos 1985). Wolf (1988) speculated that peak virus shedding may coincide with rising water temperatures and the annual spring spawning cycle resulting in inter-generational virus transmission.

Within the genus *Sprivivirus*, SVCV isolates are classified as members of the species *Carp sprivivirus*, whereas grass carp rhabdovirus (GrCRV), tench rhabdovirus (TenRV) and pike fry rhabdovirus (PFRV) isolates have been taxonomically classified to the species *Pike fry sprivivirus* (Stone et al. 2013). Carp spriviviruses have been grouped phylogenetically into 4 genogroups designated Ia to Id (Stone et al. 2003) and 2 subgroups of Ia (Iai and Iaii) (Miller et al. 2007). The genogroups correlate geographically with the area of isolation. Genogroup Ia isolates have been found in North America and China, whereas isolates of genogroups Ib to Id originate from central and eastern Europe. Virus isolation by cell culture is the most common method used for detection of SVCV and is recommended by the OIE for surveillance activities conducted to establish the SVCV-free status of susceptible fish populations (OIE 2019b). The test can detect all known SVCV genogroups, but 7–14 d are required to complete the assay (OIE 2019b).

A number of reverse-transcription quantitative PCR (RT-qPCR) assays have been developed for detection of SVCV and characterized with respect to their analytical performance (Liu et al. 2008, Yue et al. 2008, Zhang et al. 2009, Misk et al. 2016, 2017, Shao et al. 2016). The fitness of diagnostic tests for detection of SVCV can be validated to standards such as those proposed by the OIE (2019c) to ensure that their analytical and diagnostic performance is suitable for their intended purpose. A multi-phased study was designed to develop and validate 2 RT-qPCR diagnostic tests targeting SVCV nucleoprotein (Q2N) or glycoprotein (Q1G) nucleotides. Here we show that the analytical performance of these tests makes them suitable candidates for assessing their diagnostic performance. In addition, we demonstrate the utility of the tests while investigating SVCV infection dynamics in koi and testing hypotheses related to virus virulence and persistence with isolates from genogroups Ia to Id. Specifically, we hypothesized that the tests could be used to measure virus fitness traits describing SVCV virulence.

2. MATERIALS AND METHODS

2.1. Virus culture and nucleic acids

2.1.1. Viruses

The viruses used in this study are described in Table 1. Spriviviruses of carp (i.e. SVCV) and pike fry (i.e. GrCRV, TenRV) were cultured at 20°C using epithelioma papulosum cyprini (EPC) cells, whereas infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) were cultured on the same cell line at 15°C (Fijan et al. 1983, Winton et al. 2010). Cyprinid herpesvirus 3 (CyHV-3) was cultured at 15°C on common carp brain cells (Neukirch et al. 1999), while infectious pancreatic necrosis virus (IPNV) was cultured at the same tem-

Table 1. Virus isolates used in this study. Family, genus and species names accepted by the ICTV (2019) are *italicized*

Virus	Genogroup	Year, origin	Description
Alloherpesviridae, Cyprinivirus			
Cyprinid herpesvirus 3 (CyHV-3)			
F98-50[a]	European lineage	1999, USA	New York
Birnaviridae, Aquabirnavirus			
Infectious pancreatic necrosis virus (IPNV)			
Canada 3[b]	V[c]	1980, Canada	Arctic
Rhabdoviridae, Sprivivirus			
Carp sprivivirus, spring viremia of carp virus (SVCV)			
UK970469[d]	Iai	1997, UK	Asian import
980528[d]	Iai	1998, UK	Asian import
980451[d]	Iai	1998, UK	Asian import
D148[e]	Iai	2001, UK	Asian import
D120[e]	Iai	2001, UK	Asian import
E208[e]	Iaii	2002, UK	
WI[f]	Iai	2002, USA	Wisconsin
NC2002[g]	Iaii	2002, USA	North Carolina; *in vivo* study
IL[h]	Iai	2003, USA	Illinois
MO[h]	Iaii	2004, USA	Missouri
WA[h]	Iaii	2004, USA	Washington
20040741[i]	Ia	2004, China	*In vivo* study
HHOcarp06[j]	Iai	2006, Canada	Ontario
MS[k]	Ia	2007, USA	Wisconsin
20070165[i]	Ia	2007, China	*In vivo* study
OH[k]	Ia	2008, USA	Ohio
20100910[i]	Ia	2010, China	*In vivo* study
20120450[i]	Ia	2012, China	*In vivo* study
202238[p]	Ia	2016, USA	Synthetic DNA
RHV[d]	Ib	1989, Ukraine	*In vivo* study
E134[l]	Ib	2002, UK	Asian import
P4-7[d]	Ic	1983, Russia	*In vivo* study
N1-5[d]	Ic	1986, Ukraine	
S30 (aka Fijan)[m]	Id	1971, Yugoslavia	*In vivo* study
M2-78[d]	Id	1983, Moldova	
N3-14[d]	Id	1986, Ukraine	
880110[l]	Id	1988, UK	Asian import
880124[l]	Id	1988, UK	Asian import
Pike fry sprivivirus, grass carp rhabdovirus (GrCRV)			
V76[d]	II	1982, Germany	
Pike fry sprivivirus, tench rhabdovirus (TenRV)			
UK950237[d]	IV	1995, UK	
Rhabdoviridae, Novirhabdovirus			
Hirame novirhabdovirus, hirame rhabdovirus			
CA-9703[q]		1997, Korea	Synthetic DNA
Piscine novirhabdovirus, viral hemorrhagic septicemia virus (VHSV)			
99-292[n]	IVa	1999, Canada	British Columbia
CA-NB00-01[n]	IVb	2000, Canada	New Brunswick
Salmonid novirhabdovirus, infectious hematopoietic necrosis virus (IHNV)			
93-057[o]	U	1993, Canada	British Columbia
Rhabdoviridae, Perhabdovirus			
Unclassified, *Micropterus salmoides* rhabdovirus (MSRV)			
YH01[r]		2017, China	Synthetic DNA

[a]Hedrick et al. (2000); [b]Macdonald et al. (1983); [c]Nishizawa et al. (2005); [d]Stone et al. (2003); [e]Miller et al. (2007); [f]Dikkeboom et al. (2004); [g]Goodwin (2002); [h]Warg et al. (2007); [i]Emmenegger et al. (2018a); [j]Garver et al. (2007); [k]Phelps et al. (2012); [l]Way et al. (2003); [m]Fijan et al. (1971); [n]Gagné et al. (2007); [o]Garver et al. (2013); [p]Ip et al. (2016); [q]Kim et al. (2005); [r]Lyu et al. (2019)

perature on chinook salmon embryo cells (Lannan et al. 1984). All cell lines were cultured using minimal essential medium containing Hanks' salts, 2 mM L-glutamine and 2–10% fetal bovine serum (Life Technologies). Media preparations were supplemented with antibiotic/antimycotic (Life Technologies) following virus adsorption. Virus was prepared using 2 methods. The traditional virus preparations were generated by infecting cell monolayers at a multiplicity of infection (MOI) of 0.01 to 0.0001 and then harvesting monolayers when they exhibited complete cytopathic effect. These preparations were stored at −80°C as whole cell lysates. For the enriched virus preparations, cells were inoculated with SVCV HHO carp06 at an MOI of 1.0 and then harvested within 24–72 h after infection when the virus was still associated with the cells. The cell culture supernatant was removed at harvest and RNA was extracted from the infected monolayer. Virus titers were estimated using the Spearman (1908) and Kärber (1931) endpoint dilution assay (i.e. 50% tissue culture infective dose [$TCID_{50}$]) or by plaque assay (PA) (i.e. plaque-forming units [pfu]) (Batts & Winton 1989).

2.1.2. Viral nucleic acid

Nucleic acid used in the analytical validation study (i.e. ASp) was extracted from traditional virus preparations of CyHV-3, SVCV, GrCRV, TenRV, VHSV, IHNV or IPNV. CyHV-3 DNA was prepared from infected whole-cell lysates as described by Clouthier et al. (2017). Viral RNA for cDNA synthesis was extracted using the QiaAmp Viral RNA Extraction Kit (Qiagen) after clarification (2500 × *g*, 10 min, 4°C) and centrifugation (21100 × *g*, 90 min, 4°C) of infected whole-cell lysates. The semi-purified viral RNA or DNA was quantified using the Nanodrop 8000 (Nanodrop Technologies) and then stored at −80°C. Reverse transcription was performed as described by Clouthier et al. (2017) except that 100 ng of RNA were used as the template.

The enriched SVCV HHOcarp06 preparation was used as the source of RNA for creating P1 and P2 positive control samples for the Q1G and Q2N tests. TRIzol™ Reagent (ThermoFisher Scientific) was added directly to the infected monolayer in the cell culture flask immediately following removal of the cell culture medium. A cell suspension was made in the reagent by physically releasing the adherent monolayer using a cell scraper (ThermoFisher Scientific) and then homogenized with a steel bead (5 mm) and the TissueLyser (25 Hz, 2 min; Qiagen). RNA was extracted manually (ThermoFisher Scientific), resuspended in 50–200 µl molecular-grade water, measured as described in the preceding paragraph and stored at −80°C. Synthesis of cDNA was performed with RNA 10-fold serially diluted over 8 orders of magnitude (unless otherwise specified).

Tissue samples (virus free, SVCV NC2002-infected or with exogenous viral RNA from the enriched SVCV HHOcarp06 preparation) were used as the source of RNA for the RT-qPCR tests evaluated in the analytical validation study. The SVCV NC2002 exposure study with koi was described by Clouthier et al. (2021). Tissue samples were processed using TRIzol™ Reagent or TRI Reagent™ (ThermoFisher Scientific). Tissue homogenization and RNA extraction, quantitation and storage were performed as described in the preceding paragraph. Synthesis of cDNA was performed with 1500 ng genomic RNA (unless otherwise specified).

Homogenates of whole fish from the virus challenge study were prepared as described by Emmenegger et al. (2018a). The virus titer in each homogenate was evaluated using the PA test as described by Batts & Winton (1989) and expressed as pfu g^{-1} tissue. Virus titers were also determined with RNA extracted from each homogenate using TRIzol™ Reagent (ThermoFisher Scientific) and purified on Zymo-Spin columns as described by the manufacturers (Zymo Research). The RT reactions and Q2N qPCR assays were performed as described in this section and in Section 2.3. In this case, virus titer was expressed as equivalent plasmid copies (epc) g^{-1} tissue.

2.1.3. Plasmids

DNA encoding the full-length nucleoprotein or glycoprotein gene from SVCV 202238, hirame rhabdovirus (HIRRV) CA-9703 and *Micropterus salmoides* rhabdovirus (MSRV) YH01 was synthesized and inserted in the vector pJ204 (ATUM) (Table 1).

Artificial positive control (APC) plasmids (ATUM) pSVCV-APC-Q1G (2874 bp) for the Q1G test and pSVCV-APC-Q2N (2878 bp) for the Q2N test were designed using the same approach as described by Clouthier et al. (2015) (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d143p169_supp.pdf). Linearized plasmid DNA was prepared as described by Clouthier et al. (2017), except that the restriction enzyme *Nco*I (New England Biolabs) was used to cut the DNA. The purified DNA was diluted and used as P3A and P3B positive control samples for

the Q1G or Q2N tests. The plasmids each correspond to an estimated 1.87×10^6 g mol^{-1} and 3.22×10^{11} copies per µg DNA.

2.1.4. PCR and DNA sequence analysis

The Q2N region of the genome of SVCV isolate RHV was amplified by PCR using 6 pairwise combinations of the following primers: SVCV-F N691 (5′-CAT GTT GTT AAA TCA CCG GTT TGA C-3′), SVCV-F N689 (5′-GAC ATG TTG TTA AAT CAC CGG TTT GAC-3′), SVCV-R N946 (5′-GTC TTG CGT TCA GTG CTC TCT TGG AG-3′), SVCV-R N998 (5′-GCC ATC AGC AGG CTT GCA TTT G-3′) and SVCV-R N1067 (5′-GGC GGT TTT CTG TAT GTG TCT CC-3′). The RT reaction was performed as described in Section 2.1.2, and a 2.5 µl portion was used as the PCR template. The amplicons (256–379 bp) were PCR amplified, separated by electrophoresis in 2% agarose gels, gel purified, sequenced and analyzed using the conditions described by Clouthier et al. (2013) with one exception. The PCR reactions with primer SVCV-F N691 were performed using an annealing temperature of 55°C, and those with primer SVCV-F N689 were run at 57°C.

2.2. Diagnostic test development and optimization

2.2.1. Primer and probe design

Two RT-qPCR tests were constructed to bind nucleoprotein (Q2N) and glycoprotein (Q1G) nucleotides of SVCV. The tests were designed to be sensitive and species-specific, inclusive of carp spriviviruses, and exclusive of pike fry spriviviruses as well other colocalizing viruses. Viruses in Table 1 were used to evaluate the primer and probe combinations for design of the 2 RT-qPCR tests. CyHV-3 and IPNV were included since they have been detected in cyprinid hosts susceptible to SVCV infections (Dixon 2008). The novirhabdoviruses HIRRV, VHSV and IHNV and the perhabdovirus MSRV share the same virus family as carp spriviviruses, and the pike fry spriviviruses share the same genus with carp spriviviruses. Nucleotide sequences present in the National Center for Biotechnology Information GenBank database (between August 2013 and October 2014) for the nucleoprotein and glycoprotein from members of the family *Rhabdoviridae* within the genera *Sprivivirus*, *Perhabdovirus* and *Novirhabdovirus* were aligned using ClustalW (Thompson et al. 1994) and T-Coffee (Notredame et al. 2000, Di Tommaso et al. 2011) (Fig. S2) and analyzed using BioEdit v7.0.9.0 software (Hall 1999). The software programs Beacon Designer, Allele ID (Premier Biosoft International) and Primer Express (Applied Biosystems) were used to identify potential Q1G and Q2N primers and Taqman probes.

2.2.2. Primer and probe screening and optimization

qPCR primer pair combinations (Q1G n = 29; Q2N n = 14) were analyzed with SYBR Green (ThermoFisher Scientific) using 800–900 nM of each primer. Templates for the SYBR Green RT-qPCR tests were RNA extracted from whole cell lysates infected with SVCV isolates representing genotypes Ia through Id or pike fry sprivivirus isolates GrCRV or TenRV (traditional virus preparations). Amplicons were evaluated using dissociation curves. Primer pairs that failed to amplify all SVCV genotype templates or that amplified pike fry sprivivirus templates were removed from the study.

The Taqman RT-qPCR test parameters were incrementally selected using target RNA from 4 different sources: carp sprivivirus or pike fry sprivivirus-infected whole cell lysates (traditional), tissue samples containing exogenous SVCV HHOcarp06 RNA from infected whole-cell lysates (enriched), SVCV NC-2002-infected kidney tissue and plasmid DNA encoding primer and probe binding sites (pAPC-Q2N, pAPC-Q1G). Tests were optimized by selecting for their ability to (1) detect SVCV isolates representing genotypes Ia through Id, (2) not detect pike fry sprivivirus isolates GrCRV or TenRV and (3) amplify plasmid DNA from $10^{7.7}$ to $10^{0.7}$ copies per reaction.

The iterative process of identifying the optimum primer and probe concentrations was described by Clouthier et al. (2015). Briefly, primer concentrations from 100 to 1000 nM were tested in 100 nM increments, and those producing the lowest quantification cycle (Cq) (Bustin et al. 2009) value with SVCV isolates and no Cq value with pike fry sprivivirus isolates were selected for further analysis. For this part of the study, the probe concentration was maintained at 250 nM. Probe concentrations from 100 to 250 nM were then tested in increments of 50 nM, and those producing the highest fluorescence with representative SVCV isolates and no fluorescence with pike fry sprivivirus isolates were selected for further analysis. The effects of changing the annealing temperature (60 to 61°C) and adding an extension step (72°C for 30 s per cycle) were also evaluated.

The optimum quantity of RNA to use in the Q2N and Q1G tests was identified by adding a range of RNA (i.e. 100, 250, 500, 750, 1000, 2000, 3000, 4000 and 5000 ng) extracted from either naïve carp kidney (Clouthier et al. 2017) or koi brain tissue (Clouthier et al. 2020) seeded with 50 ng exogenous SVCV HHO-carp06 viral RNA from infected whole cell lysates (enriched).

2.3. RT-qPCR tests Q1G and Q2N

The Q2N test targets nucleoprotein-encoding nucleotides 854–950 (97 bp product) of GenBank accession number U18101 (Fig. S2). With the exception of SVCV isolate RHV, no base mismatches were observed in the SVCV N DNA sequences corresponding to the primer and probe binding sites (Fig. S2A). The primers and probes selected for the Q2N test were forward primer SVCV F2 (5′-CTG GAC AGG AGA TAG ATA A-3′), reverse primer SVCV R6 (5′-GAC GGA TTC TTT ATT GTT G-3′) (MilliporeSigma), Q2N assay probe SVCV P6 (5′-6FAM-CTC TGC CAA ATC ACC AT-MGB-3′) and positive control pAPC probe APC-P (5′-VIC-ACC GTC TAG CAT CCA GT- MGBNFQ-3′) (Fig. S1A) (ThermoFisher Scientific). Each 25 µl reaction contained 800 nM SVCV F2, 800 nM SVCV R6, 200 nM SVCV P6, 1× Taqman Universal PCR Master Mix (Applied Biosystems) and 1 µl cDNA (optional 150 nM APC-P).

The Q1G test targets glycoprotein-encoding nucleotides 3643–3735 (93 bp product) of GenBank accession number U18101 (Fig. S2). Base mismatches were present in the primer and/or probe binding sites for most of the SVCV G DNA sequences (Fig. S2B). The primers and probes selected for Q1G test were forward primer SVCV F1400 (5′-ATT TGG ATC ACA GAT GAG AC-3′), reverse primer SVCV R400-11 (5′-ATC CCC TCT CGG AGC ATT CCC GTA CA-3′) (MilliporeSigma), Q1G assay probe SVCV P1000 (5′-6FAM-TAG AGG AAG TTG AAG GAA TT-MGB-3′) and the same pAPC probe APC-P described for the Q2N test (Fig. S1B) (ThermoFisher Scientific). Each 25 µl reaction contained 900 nM SVCV F1400, 900 nM SVCV R400-11, 200 nM SVCV P1000, 1× Taqman Universal PCR Master Mix (Applied Biosystems) and 1 µl cDNA (optional 200 nM APC-P).

The sample set for each RT-qPCR test run included positive and negative control samples that were used to validate the results obtained from nucleic acid extraction through to RT and qPCR. The Q1G and Q2N control material consisted of an RNA resuspension buffer blank (N1), 50 mg kidney tissue seeded with 0.466 ng RNA from the SVCV HHOcarp06 enriched preparation (P1), water (N2), 8.8×10^{-3} ng RNA from the SVCV HHOcarp06 enriched preparation (P2), water (N3) and 5×10^4 (P3A; high copy number) and 5×10^2 (P3B; low copy number) copies of pSVCV-APC-Q1G per reaction or 5×10^5 (P3A; high copy number) and 5×10^3 (P3B; low copy number) copies of pSVCV-APC-Q2N per reaction.

The control samples were added at the RNA extraction (N1, P1), RT (N2, P2) and the qPCR steps (N3, P3A, P3B) of each test. Since each set of control samples was run in parallel with unknown samples, the negative control samples were used to detect false positive results arising from cross-contamination between samples or from the positive control samples. The latter were used to detect false negative results such as those arising from machine malfunction or technical errors.

The P3 synthetic plasmid constructs pSVCV-APC-Q2N and pSVCV-APC-Q1G each contain a binding site for the VIC-labeled probe APC-P, which can be added to each reaction mix (Fig. S1). As such, the Q2N and Q1G tests were designed to have 2 probes present in each qPCR reaction, with each probe labeled with a different fluorophore and recognizing a different target. The FAM-labeled probes P6 (Q2N) and P1000 (Q1G) bind with SVCV sequences found in the test sample or P3 plasmids pSVCV-APC-Q2N or pSVCV-APC-Q1G (Fig. S1). The VIC-labeled probe APC-P only recognizes sequences found in the P3 plasmids (Fig. S1) and can be used to detect false positive results arising from cross-contamination of a test sample with P3A or P3B.

The qPCR tests were run in a 96-well format with replicates of 3–6 per sample on the Stratagene Mx3000/5P qPCR platform using the thermocycling profile of 1 cycle of 2 min at 50°C and 1 cycle of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The Cq values were reported after manually adjusting the threshold to exceed the background level and be in the linear phase of the amplification curve. The copy number equivalent (i.e. equivalent plasmid copies, epc) present in each test sample was determined using the standard curve method with plasmids pSVCV-APC-Q1G or -Q2N.

2.4. Analytical validation

2.4.1. Analytical sensitivity

The analytical sensitivity of Q1G and Q2N was assessed with standard curves produced using (1)

plasmid DNA (pSVCV-APC-Q1G, pSVCV-APC-Q2N), (2) RNA extracted from whole cell lysates of EPC monolayers infected with SVCV HHOcarp06 or (3) RNA extracted from SVCV NC2002-infected kidney tissue. The reaction efficiency with each template was evaluated to determine if SVCV in infected tissue could be quantified using plasmid DNA as a proxy. Test parameters of reaction efficiency and ASe were also used to determine if host RNA (1500 ng per reaction) or the APC probe (150 or 200 nM per reaction) altered assay performance. Nucleic acid was serially diluted 2-, 5- or 10-fold, and RT-qPCR standard curves were constructed from each dilution analyzed in replicates of 3, 5 or 6 (5 dilution series with 6 replicates per dilution assessed in 5 independent qPCR runs). The concentration of nucleic acid in which 50 or 100% of the replicates were detected (<40 Cq) was identified as the corresponding limit of detection (LOD) and was expressed as copies of plasmid DNA.

2.4.2. Analytical specificity

The exclusivity of Q1G and Q2N was determined using nucleic acid extracted from cell lysates (traditional virus preparation) infected with pike fry sprivivirus, novirhabdovirus, cyprinivirus or aquabirnavirus (n = 7; Table 1) as well as plasmid DNA (10^6 copies) encoding the full-length N or G gene from novirhabdovirus HIRRV or perhabdovirus MSRV (n = 2; Table 1). The inclusivity of these assays was investigated using RNA isolated from cell lysates (traditional virus preparation) infected with carp spriviviruses (n = 22; Table 1) as well as plasmid DNA encoding the full-length N or G gene from SVCV 202238 (n = 1; Table 1). Infected cell lysates were normalized to 10^6 $TCID_{50}$ ml^{-1}. The RT was performed with 100 ng RNA extracted from 1 ml of this material (see Section 2.1 for RT and RNA extraction methods). The Q2N and Q1G tests were run in triplicate per sample using the parameters described in Section 2.3. The mean Cq values were reported so that the relative sensitivity of Q2N and Q1G with each isolate could be evaluated. The inclusivity of Q2N and Q1G was also evaluated using tissues from koi exposed to SVCV isolates 20040741, 20070165, 20100910, 20120450 or S30 (n = 5; Table 1). In this case, the results were reported as detected or not detected. Using the binary results from all 37 viruses, inclusivity and exclusivity estimates were generated using 2-way cross-tabulation tables (Table 2) as the level of agreement between expected and observed results (with 95% confidence intervals). For Q2N and Q1G, inclusivity represents the ability to detect SVCV isolates and exclusivity represents the degree to which the assay did not detect viruses outside of the carp spriviviruses.

Table 2. Table used for determining analytical specificity of the Q1G and Q2N tests. Inclusivity = A/(A + B); Exclusivity = D/(C + D)

	Observed		
	Positive	Negative	Total
Expected			
Positive	A	B	A + B
Negative	C	D	C + D
Total	A + C	B + D	A + B + C + D

2.4.3. Analytical repeatability

The intra- and inter-assay repeatability of Q1G and Q2N was tested in 5 runs with plasmid DNA (Q1G, pSVCV-APC-Q1G; Q2N, pSVCV-APC-Q2N) 10-fold serially diluted from $10^{8.7}$ to 5, and 2-fold serially diluted from 25 to 0.78 copies per reaction (6 replicates per dilution). Continuous outcome data from the 2 tests for positive control samples P1, P2, P3A and P3B was also evaluated for repeatability using sample results from 1 batch of P1, P3A and P3B and 2 batches of P2 assessed in 31–69 independent runs performed by 3 or 4 analysts on 1 or 2 qPCR platforms (Stratagene Mx3000/5P; Applied Biosystems StepOne Plus) in 3 or 4 laboratories over a 3 yr period. The Q2N and Q1G tests were run using the parameters described in Section 2.3. The results were presented graphically by plotting the mean Cq of the replicates against the standard deviation. Continuous outcome data (i.e. Cq values) variability was estimated as a ratio of the SD to the mean (i.e. coefficient of variation, CV). Statistical data analyses were implemented in StataI/C (v15.1).

2.5. SVCV persistence in koi

2.5.1. Fish and virus exposure

Shusui koi (10.5 mo, 3.4 g average weight, 6.6 cm average length) were initially held at 16–18°C in the aquatic laboratory at the US Geological Survey Western Fisheries Research Center (WFRC; Seattle, Washington). The koi were diagnosed as negative for

CyHV-3 and SVCV prior to shipping. Fish were fed on alternate days with a mixed feed diet of Hikari Gold mini pellets (Kyorin Food Industries) and Classic Fry 1.5 mm floating pellets (Skretting). Immediately prior to challenge, fish were transferred on site to the aquatic biosafety level 3 laboratory where they were housed in 30 l tanks containing water at 10–11°C. Quadruplicate groups of 21 fish were challenged by immersion for 1 h in 3 l aerated, static water containing SVCV (10^5 pfu ml^{-1}) (Ia: 20040741, 20070165, 20100910, 20120450, NC2002; Ib: RHV; Ic: P4-7; Id: S30 [also known as Fijan]) or were mock-challenged in water containing an equivalent volume of cell culture media. Continuous water flow was restored and water temperature was increased 1°C d^{-1} to 13–14°C and held at that temperature. The fish were monitored daily for 35 d. Dead fish were removed on the day of death and immediately frozen at −80°C. The isolates and dose of SVCV were selected based on the virus exposure work of Emmenegger et al. (2018b), in which acute SVC disease was observed in younger Beni Kiko or Sanke koi (3.5 mo). Cumulative percent mortality (cpm) was determined in triplicate as the number of fish that died per tank divided by the total number of fish × 100 and was reported as an average of the 3 tanks. A significant relationship was designated for cpm comparisons yielding p-values of ≤0.05 using a 1-way ANOVA and, if applicable, a Tukey-Kramer multiple-comparison test. This analysis was performed in GraphPad Prism 8 software. Mean day of death (MDD) values were calculated as the sum of the day(s) of death post-exposure divided by the total number of dead fish per tank. The average MDD for replicate tanks is reported. The study protocol was approved by the WFRC Institutional Animal Care and Use Committee and the WFRC Institutional Biosafety Committee under the guidelines provided in the Guide for the Care and Use of Laboratory Animals (NRC 2011).

2.5.2. Targeted sampling

Live fish (n = 2) were removed 1, 3, 5, 7, 10, 14 and 17 d post exposure (dpe) from the fourth tank, euthanized and immediately frozen at −80°C. Fish that survived the 35 d challenge were pooled according to treatment and held in duplicate 30 l tanks. Live fish (n = 2; 1 per tank) were removed 35, 49, 63, 77 and 84 dpe, euthanized and immediately frozen at −80°C. At 93 dpe, fish were transferred to duplicate 10 l tanks containing water at 10–11°C. Water temperature was increased 1°C d^{-1} to 13–14°C and held at that temperature. Live fish (n = 2 or 3; 1 or 2 per tank) were removed 133 and 149 dpe, euthanized and immediately frozen at −80°C. Any fish remaining at 149 dpe were used as donor fish in a cohabitation exposure study in which quadruple groups of 4 donor Shusui koi (i.e. convalescent) were held with 14 naïve Sanke koi fry (1.5 mo, average weight 1.54 g, average length 4.6 cm) in 8 l tanks containing water at 10–11°C. The donor koi were anaesthetized in buffered tricaine methanesulfonate (MS222), and a portion of the left pelvic fin was removed to distinguish them from the naïve koi. Water temperature was increased 1°C d^{-1} to 13–14°C and held at that temperature. Live donor fish (n = 4; 1 per tank) were removed 17 d into the cohabitation exposure (i.e. 167 dpe of the donor koi), euthanized and immediately frozen at −80°C. Fish were euthanized by an overdose of buffered MS222. Virus titer in each dead or sampled fish was determined as described in Section 2.1.

Additional fish were sampled over the course of this study, but an equipment failure of the −80°C freezer compromised the samples and they were discarded. The samples described above had been processed prior to the mechanical failure with the exception of the following time point samples: 133 dpe, koi exposed to isolates 20040741, NC2002 or Fijan; 149 dpe, koi exposed to isolates 20040741, NC2002, Fijan, 20120450. These 14 fish are missing from the dataset.

2.5.3. Statistical methods for analyzing Q2N and PA diagnostic test results

Ordinary least-squares linear regression was performed to assess the significance (determined at $p < 0.05$) and relationship between variables.

The proportion of agreement and Cohen's kappa (Cohen 1960) statistics were used to estimate inter-test and inter-fish concordance of Q2N and PA binary test outcomes. Results were interpreted according to Landis & Koch (1977), and confidence intervals for kappa were calculated as described by Reichenheim (2004). Differences in the relative number of positive tests obtained by each test within a test pair were considered statistically significant if the p-value for McNemar's test statistic (χ^2) was ≤0.05 (Dohoo et al. 2009).

Concordance correlation coefficient (Lin 1989, 2000) and Bland and Altman limits of agreement (Bland & Altman 1986, Barnhart et al. 2007) were used as described previously (Clouthier et al. 2017)

to evaluate the statistical agreement in virus titer results obtained with the Q2N and PA tests. Virus titer agreement within and between the 2 tests is presented as point estimates as well as graphically to identify patterns within the data.

All statistical analyses described in this section were performed in Stata/IC (v15.1).

3. RESULTS

3.1. RT-qPCR Q2N and Q1G

The Q2N and Q1G tests can be run with or without the VIC-based probe APC-P included in the reaction mix. The Q2N and Q1G FAM-based tests, when duplexed with the internal positive control VIC-based probe APC-P, had similar reaction efficiencies to the respective VIC-based test (Fig. S3). The relative slopes of the qPCR standard curves for each test varied by less than 0.1 relative to the corresponding internal APC test (Fig. S3). The assays were linear across 9 orders of magnitude ($10^{8.7}$ to $10^{0.7}$ copies) with a strong correlation between cycle number and dilution factor (R^2 = 0.995 to 0.998, Fig. S3). In the absence of the VIC-labeled probe, amplification efficiencies of Q2N and Q1G qPCR reactions containing plasmid or SVCV-infected tissue or cell lysate RNA serially diluted 10-fold ranged from −3.3 to −3.6 (Fig. S4). The similarities in reaction efficiencies independent of template type showed that plasmid DNA could be used to define the LOD for Q2N and Q1G. The poor reaction efficiency of the duplex Q2N/APC-P test (i.e. −4.1) suggested that Q2N test should not be multiplexed with the APC-P probe (Fig. S3A).

The RT-qPCR tests Q2N and Q1G could be performed with up to 5000 ng total tissue RNA added to the RT reaction before interference in test performance was observed (data not shown). We typically add 1500 ng template RNA from brain or kidney tissue to the RT reaction.

3.2. Analytical validation

Plasmids pSVCV-APC-Q2N and pSVCV-APC-Q1G were used to determine the ASe for Q2N and Q1G (Fig. S5). The tests were positive with plasmid DNA present between $10^{8.7}$ and $10^{0.7}$ copies, or 8 or 9 orders of magnitude (Fig. S5). The observed 50% LOD for Q2N and Q1G was less than 3.13 copies of pSVCV-APC-Q2N (28 of 30 replicates tested positive; 36.56 ± 1.30 [SD] Cq; Fig. S5B) or pSVCV-APC-Q1G (21 of 30 replicates tested positive; 37.11 ± 2.10 Cq; Fig. S5D). The 100% LOD for Q2N was 5 copies of pSVCV-APC-Q2N (35.53 ± 1.00 Cq) and for Q1G, it was 12.5 copies of pSVCV-APC-Q1G (34.39 ± 1.64 Cq). The presence of host RNA (1500 ng) did not significantly alter the LOD of either test, nor did the presence of the VIC-labeled probe APC-P in Q1G reactions (data not shown). The poor reaction efficiency of the duplex Q2N/APC-P test resulted in a significant shift in the LOD from 3.13 to 25 (50% LOD) and from 5 to 50 (100% LOD) copies (data not shown). The results indicated that the Q2N test should not be duplexed with the APC-P probe.

The ASp of Q2N and Q1G was determined using binary data (detected/not detected) to evaluate the level of agreement between the observed and expected status with the panel of viruses in Table 1. The carp sprivivirus isolates included representatives from the 4 genogroups Ia to Id. The inclusivity (including 95% confidence intervals) of Q2N was 100% (88–100%) whereas for Q1G, it was 96% (82–100%), as the test did not detect SVCV isolate 20120450. The relative ability (i.e. Cq value) of Q2N and Q1G to amplify different genogroups was evaluated with a subset of 22 carp sprivivirus isolates (Fig. 1). All of the isolates were detected within 21 cycles by Q2N except for RHV (24.73 Cq), and for Q1G, the exceptions were RHV (24.13 Cq) and E134 (27.06 Cq) from genogroup Ib and 880110 (27.05 Cq) and 880124 (28.11 Cq) from genogroup Id (Fig. 1). The exclusivity of Q2N was 89% (52–100%) and for Q1G, it was 100% (66–100%). Q2N and Q1G did not recognize CyHV-3, IPNV, HIRRV, VHSV, IHNV or MSRV, and Q1G did not detect either pike fry sprivivirus tested (Table 1). Positive Q2N test results with an average Cq value of 36.21 were obtained with a pike fry sprivivirus isolate, V76 (Fig. 1).

Repeatability of Q1G and Q2N was investigated using Cq values obtained from 5 independent runs with plasmid DNA serially diluted from $10^{8.7}$ to $10^{-0.11}$ copies per reaction (6 replicates per dilution) (Fig. 2). The Cq values obtained for each dilution were also used to generate point estimates of variability expressed as the CV. The average intra-assay CV varied from 0.74 to 9.64 (Fig. 2) and the inter-assay CV ranged from 1.14 to 9.45 (Fig. 2). The widest dispersion of Cq values from the mean was observed with samples containing the highest plasmid copy number of $10^{8.7}$ copies (Fig. 2).

Repeatability of Q2N and Q1G was also investigated using Cq values obtained with positive control samples P1, P2, P3A and P3B (Fig. S6). The within-

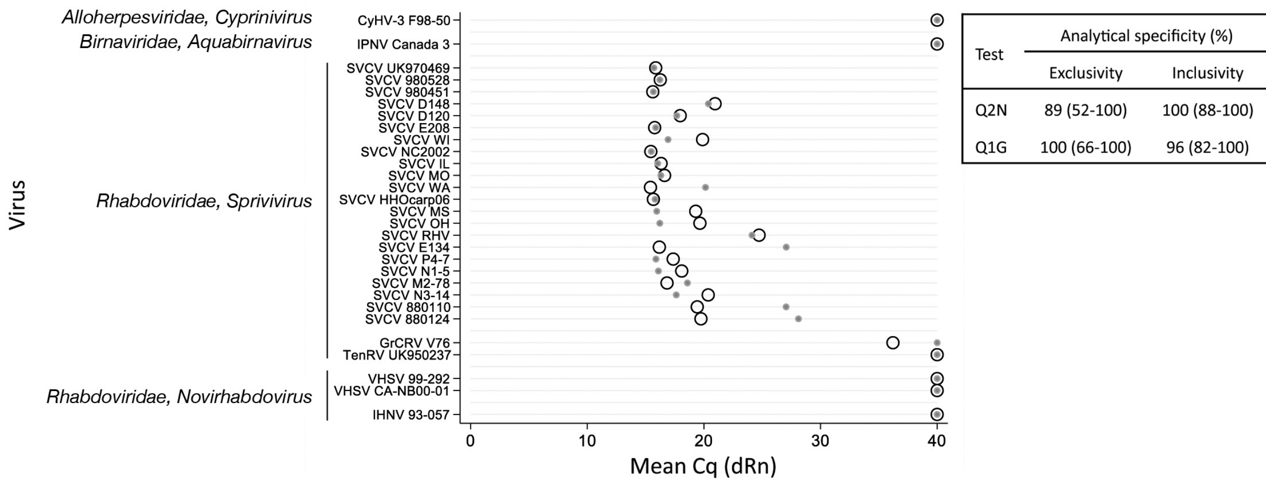


Fig. 1. Analytical specificity of spring viremia of carp virus diagnostic tests Q2N and Q1G. The relative specificity of the tests Q2N (○) and Q1G (●) is demonstrated by differences in the mean quantification cycle (Cq) values obtained with and between select viruses (Table 1). The inclusivity and exclusivity of Q2N and Q1G (table inset) was determined using the binary test results defined as detected (<40 Cq) or not detected (40 Cq). dRn: ROX (carboxy-X-rhodamine)-normalized, baseline corrected fluorescence view

assay CV calculated on average for P1 was 1.16 for Q2N and 1.07 for Q1G, whereas the inter-assay CV was 4.66 for Q2N and 4.44 for Q1G (Fig. S6). The within-assay CV calculated on average for P2 was 1.28 for Q2N and 0.98 for Q1G whereas the inter-assay CV was 6.12 for Q2N and 6.60 for Q1G (Fig. S6). The average intra-assay CV calculated for P3A and P3B varied from 1.11 to 1.78, whereas the inter-assay CV values ranged from 2.87 to 4.79 for both tests (Fig. S6).

3.3. SVCV exposure of koi

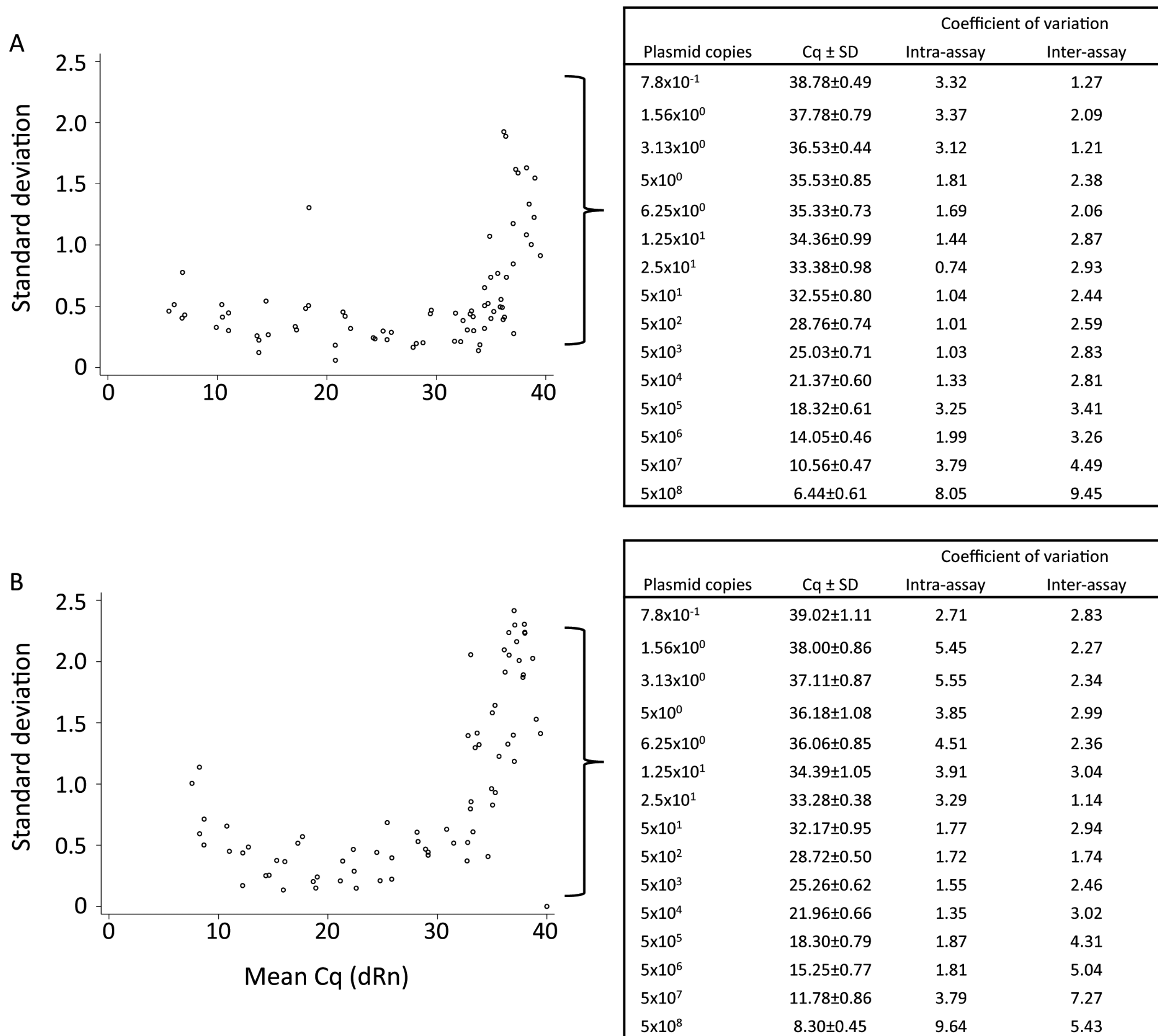
The exposure study was designed to investigate SVCV infection dynamics across genogroups in koi. The first part of the study, Days 0 through 35, was designed to measure the relative virulence (i.e. cumulative mortality) of 8 isolates of SVCV belonging to genogroups Ia to Id in koi, and the goal of the second part of the study, Days 0 through 167, was to measure the relative persistence (i.e. virus presence) of SVCV in koi. The virulence categories of each isolate were based on mortality and were established by Emmenegger et al. (2018b).

Cumulative mortality at the end of the 35 d study ranged from 3 to 26% (Fig. 3A) and was not significantly ($p < 0.05$) different between treatments except for fish exposed to high virulence isolate 20040741 versus mock-infected fish and groups of fish exposed to the low virulence isolates RHV, 20100910 and 20070165. Of the virus-exposed fish that died, 60% (9/15) displayed signs of disease, and of these, 44% (4/9) were exposed to highly virulent isolates. Clinical signs were first evident in fish exposed to highly virulent isolates and appeared 7–10 d following virus exposure. The MDD for the high virulence (i.e. 17 [NC2002], 23 [20040741] or 20 [Fijan] dpe), moderate virulence (i.e. 18 [20120450] or 24 [P4-7] dpe) and low virulence isolates (i.e. 20 [20070165], 15 [20100910] or 24 [RHV] dpe) suggested that the peak infection phase of the study occurred within the first 20 d following exposure (Fig. 3A). All of the fish that died in the first 35 d after virus exposure (n = 15) were positive by PA, with titers ranging from $10^{5.51}$ to $10^{7.12}$ pfu g^{-1} tissue. Virus was not detected in the 3 mock-challenged fish that died.

A targeted time point sampling study was undertaken in koi between 1 and 167 dpe following mock exposure or static immersion in 1 of 8 SVCV isolates. For reference, whole body homogenates from 2 to 4 fish per virus isolate, per time point at 1, 3, 5, 7, 10, 14, 17, 35, 49, 63, 77, 84, 133, 149 and 167 dpe (n = 290) and from dead fish (n = 29) were tested for virus presence and titer using the PA and Q2N tests. The cumulative mortality is presented in Fig. 3B and virus presence and titer results are presented in Fig. 4.

Virus was detected by 1 or both tests in 83% (24/29) of fish that died on non-sampling days (Fig. 4). The Q2N test detected SVCV in more samples relative to the PA test (Q2N, 79% [23/29]; PA, 38% [11/29]). Virus titers ranged from $10^{2.7}$ to $10^{5.9}$ pfu g^{-1} tissue by PA and $10^{3.9}$ to $10^{9.2}$ epc g^{-1} tissue by qPCR. Virus was not detected in any of the mock-challenged fish.

Virus titer decreased significantly ($p < 0.05$) between 1 and 167 dpe (Fig. 4). Titer decreased daily



(A)

Plasmid copies	Cq ± SD	Coefficient of variation Intra-assay	Inter-assay
7.8×10^{-1}	38.78±0.49	3.32	1.27
1.56×10^{0}	37.78±0.79	3.37	2.09
3.13×10^{0}	36.53±0.44	3.12	1.21
5×10^{0}	35.53±0.85	1.81	2.38
6.25×10^{0}	35.33±0.73	1.69	2.06
1.25×10^{1}	34.36±0.99	1.44	2.87
2.5×10^{1}	33.38±0.98	0.74	2.93
5×10^{1}	32.55±0.80	1.04	2.44
5×10^{2}	28.76±0.74	1.01	2.59
5×10^{3}	25.03±0.71	1.03	2.83
5×10^{4}	21.37±0.60	1.33	2.81
5×10^{5}	18.32±0.61	3.25	3.41
5×10^{6}	14.05±0.46	1.99	3.26
5×10^{7}	10.56±0.47	3.79	4.49
5×10^{8}	6.44±0.61	8.05	9.45

(B)

Plasmid copies	Cq ± SD	Coefficient of variation Intra-assay	Inter-assay
7.8×10^{-1}	39.02±1.11	2.71	2.83
1.56×10^{0}	38.00±0.86	5.45	2.27
3.13×10^{0}	37.11±0.87	5.55	2.34
5×10^{0}	36.18±1.08	3.85	2.99
6.25×10^{0}	36.06±0.85	4.51	2.36
1.25×10^{1}	34.39±1.05	3.91	3.04
2.5×10^{1}	33.28±0.38	3.29	1.14
5×10^{1}	32.17±0.95	1.77	2.94
5×10^{2}	28.72±0.50	1.72	1.74
5×10^{3}	25.26±0.62	1.55	2.46
5×10^{4}	21.96±0.66	1.35	3.02
5×10^{5}	18.30±0.79	1.87	4.31
5×10^{6}	15.25±0.77	1.81	5.04
5×10^{7}	11.78±0.86	3.79	7.27
5×10^{8}	8.30±0.45	9.64	5.43

Fig. 2. Analytical repeatability of the (A) Q2N and (B) Q1G tests. Quantification cycle (Cq) values for the FAM-labeled probes were generated with plasmid pSVCV-APC-Q2N or pSVCV-APC-Q1G DNA diluted from $10^{8.7}$ to 0.78 copies (dRn: ROX-normalized, baseline corrected fluorescence view). Measurements were performed in replicates of 6 in 5 independent runs. Intra- and inter-assay coefficient of variation for Cq values as well as the mean ± SD Cq values for each of 5 runs are presented for each dilution in the tables, where Cq is the mean of the mean Cq per run, SD is the mean of the mean Cq SD per run, and the intra-assay CV is the average of the within-assay coefficient of variation for all runs

on average by $10^{0.03}$ epc g^{-1} tissue or $10^{0.04}$ pfu g^{-1} tissue. Virus was detected by 1 or both assays in at least 1 of the time point samples collected for each isolate in the first 35 dpe with the exception of Day 1. In the latter case, both assays tested negative with 5 of the 8 SVCV strains. Positive test results on Day 1 were obtained with isolates 20070165 and 20120450 (Q2N) and NC2002 (PA). RHV was detected by PA but not Q2N in fish collected at 3, 5, 7, 10, 63 and 77 dpe.

Virus prevalence measured by Q2N or PA increased significantly ($p < 0.05$) according to virulence category in fish sampled from 1 to 167 dpe (Fig. 4). For every change in virulence category from low to moderate and moderate to high, virus prevalence increased by 20% (Q2N) or 10% (PA).

Virus titer measured by Q2N or PA increased significantly ($p < 0.05$) according to virulence category in fish sampled from 1 to 167 dpe (Fig. 4). For every change in virulence category from low to moderate

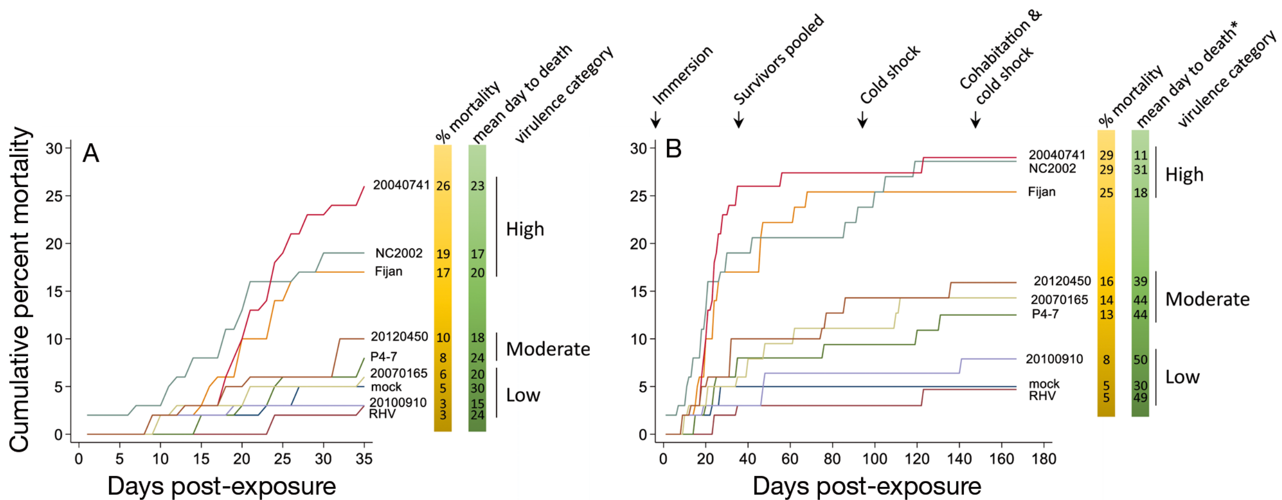


Fig. 3. Koi *Cyprinus carpio* L. exposed to spring viremia of carp virus (SVCV). The cumulative percent mortality and mean day to death are reported for groups of koi (3.4 g) (A) 35 d or (B) 167 d following immersion in no virus (mock) or 10^5 plaque-forming units per ml of SVCV isolates RHV, P4-7, Fijan, NC2002, 20040741, 20120450, 20070165 or 20100910. Virulence categories were based on mortality and were established by Emmenegger et al. (2018b)

and moderate to high, virus titer increased by $10^{0.5}$ epc g^{-1} tissue or $10^{0.4}$ pfu g^{-1} tissue.

A significant ($p < 0.05$) and positive relationship was observed between the maximum titer determined by Q2N or PA for each isolate and its virulence ranking (Fig. 4). For every change in virulence category from low to moderate and moderate to high, the maximum virus titer increased by $10^{0.9}$ epc g^{-1} tissue or $10^{0.4}$ pfu g^{-1} tissue in fish sampled from 1 to 167 dpe.

A significant ($p < 0.05$) and negative relationship was found for the time required to reach maximum virus titer and the virulence ranking of SVCV isolates (Fig. 4). For every change in virulence category from low to moderate and moderate to high, maximum virus titer determined using the PA test was obtained on average 2.5 d earlier.

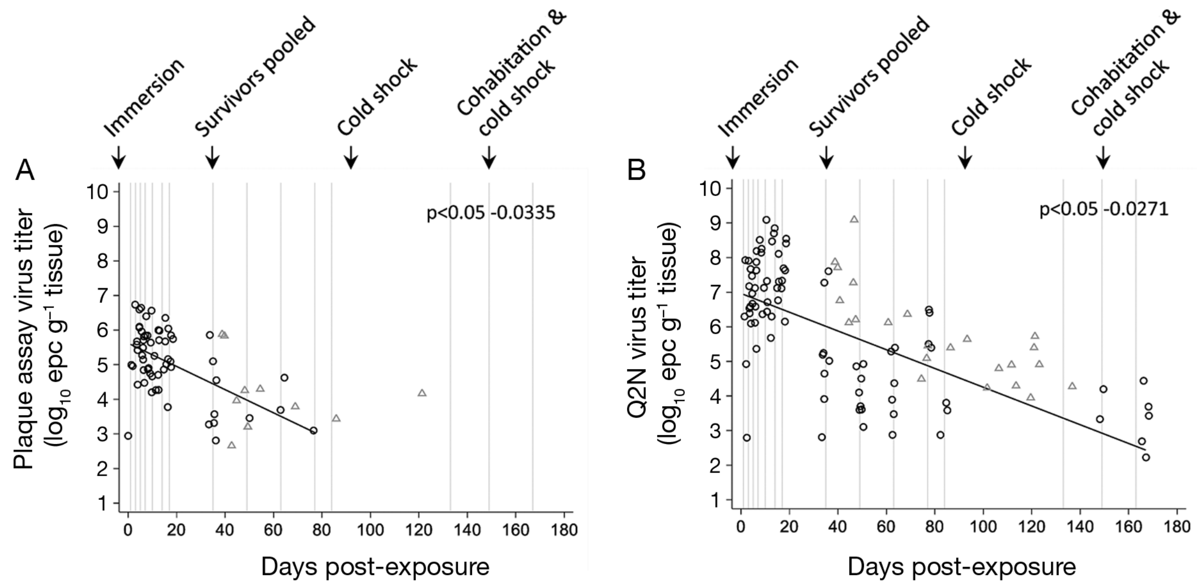
The Q2N test but not the PA test detected virus after 77 dpe (Fig. 4). At 167 dpe, all 3 highly virulent isolates were detected by Q2N, and the percentage of positive fish was higher (58 %, 7/12) relative to the groups of fish infected with the moderate (25 %, 2/8) or low (8 %, 1/12) virulence isolates. No significant difference ($p > 0.05$) in virus titer was observed between genogroups using either test.

Agreement for binary outcomes (i.e. virus detected or not detected) for the PA and Q2N tests is reported in Table 3. Agreement of paired test results for duplicate fish (n = 2 × 127) ranged from 77 (Q2N) to 87 % (PA) (Table 3). Agreement between Q2N and PA test results was 71 % for fish (n = 128) sampled from 1 to 167 dpe (Table 3). Agreement increased to 87 % when data were used from a subpopulation of fish with virus titers within the LOD of the PA (i.e. fish sampled from 1 to 17 dpe; data not shown). Within a sample set, sample pairs testing positive ranged in number from 51 for the Q2N test, 43 for the PA test and 52 between PA and Q2N (Table 3). Kappa estimates for paired test results were in the range of moderate to substantial categories for Q2N (0.54) and PA (0.74) and in the moderate agreement category between Q2N and PA (0.43). In the latter case, McNemar's test was significant ($p < 0.05$) (Table 3).

Estimates of agreement for SVCV titer results from concordance correlation coefficients were 0.803 for Q2N, 0.249 for PA and 0.301 between Q2N and PA (Table 4). A concordance correlation plot of paired virus titer results from Q2N and PA is shown in Fig. 5A. The Bland-Altman average difference for pairwise comparisons of $\log_{10}$ virus titer values was 0.054 for Q2N, −0.154 for PA and −1.783 between Q2N and PA (Table 4). Virus titers estimated by the Q2N test were significantly higher ($p < 0.05$) compared to those of the PA test. A Bland and Altman plot for test pair results reported for Q2N and PA is presented in Fig. 5B.

4. DISCUSSION

The analytical performance characteristics of 2 SVCV-specific RT-qPCR tests, Q1G and Q2N, were validated according to international standards (OIE 2019d). The performance estimates for both tests



Virus category	SVCV isolate	Virus positive fish 1 to 167 dpe (%)		Maximum titer (log10 pfu or epc g^{-1} tissue)		Time to maximum titer (dpe)		Maximum positive time point (dpe)*
		PA*	Q2N*	PA*	Q2N*	PA*	Q2N	
High	20040741	50.00 (14/28)	82.14 (23/28)	$10^{6.3}$	$10^{8.7}$	7	14	167
	NC2002	57.14 (16/28)	85.71 (24/28)	$10^{6.7}$	$10^{8.6}$	7	17	167
	Fijan	50.00 (14/28)	67.86 (19/28)	$10^{6.6}$	$10^{9.1}$	10	10	167
Moderate	20120450	40.00 (12/30)	56.67 (17/30)	$10^{5.7}$	$10^{7.7}$	17	14	63
	P4-7	43.75 (14/32)	62.50 (20/32)	$10^{6.6}$	$10^{8.8}$	3	14	167
Low	20070165	30.30 (10/33)	60.61 (20/33)	$10^{5.7}$	$10^{7.6}$	5	5	167
	20100910	28.13 (9/32)	40.63 (13/32)	$10^{5.8}$	$10^{7.3}$	17	17	49
	RHV	40.63 (13/32)	12.50 (4/32)	$10^{5.9}$	$10^{6.2}$	17	17	49

Fig. 4. Koi *Cyprinus carpio* L. exposed to spring viremia of carp virus (SVCV). (A) Plaque assay (PA) test results expressed as $\log_{10}$ plaque-forming units (pfu) g^{-1} tissue and (B) Q2N test results expressed as $\log_{10}$ equivalent plasmid copies (epc) g^{-1} tissue. Results are displayed for fish sampled 1, 3, 5, 7, 10, 14, 17, 35, 49, 63, 77, 84, 133, 149 and 167 d post exposure (dpe) (i.e. time point fish, ○) and fish that died on non-sampling days (i.e. dead fish, △). Virus titers for time point fish are the average of 2–4 fish collected per time point and virus isolate. A random jitter was added to expose the density of the data. Linear regression lines (solid black) and statistics are derived from time point data (1–167 dpe) indicated by grey vertical lines. Table inset presents the percent of fish that tested positive at 167 dpe, maximum virus titer and time to maximum titer obtained with the PA and Q2N tests. The maximum positive time point is the last time point at which virus was detected with either test. *Linear regression $p < 0.05$ by virulence category

Table 3. Agreement of paired binary test results. Fish (n = 2) were collected at time points 1, 3, 5, 7, 10, 14, 17, 35, 49, 63, 77, 84, 133, 149 and 167 d post exposure. Test pairs indicate fish (fish 1, fish 2) and assay (PA: plaque assay; Q2N: RT-qPCR test). Symbols in parentheses describe the test results presented for test 1 and test 2, respectively. Estimates of between-fish or between-test agreement for binary outcomes were generated using the proportion of agreement and Cohen's kappa methods. Confidence intervals (95%) accompany each kappa estimate. McNemar's test was significant for pairs of test results denoted with an asterisk (*)

Test pairs (test 1, test 2)	2 × 2 contingency table (+,+)	(+,–)	(–,+)	(–,–)	McNemar's chi-squared p-value	Agreement (%)	Cohen's kappa
Q2N							
Fish 1, Fish 2	51	16	13	47	0.7111	77.17	0.5431 (0.397, 0.689)
PA							
Fish 1, Fish 2	43	8	8	68	1.0000	87.40	0.7379 (0.618, 0.858)
PA & Q2N							
PA, Q2N	52	7	30	39	0.0002*	71.09	0.4343 (0.290, 0.579)

Table 4. Agreement of paired virus titer test results. Fish were collected at time points 1, 3, 5, 7, 10, 14, 17, 35, 49, 63, 77, 84, 133, 149 and 167 d post exposure. Test pairs indicate fish (fish 1, fish 2) and assay (PA: plaque assay; Q2N: RT-qPCR test). Confidence intervals (95%) accompany each estimate

Test pairs (test 1, test 2)	Concordance correlation coefficient (Degree of agreement)	Bland Altman's limit of agreement (Average difference)	Tests positive in both sample sets
Q2N			
Fish 1, Fish 2	0.803 (0.681, 0.882)	0.054 (−1.908, 2.016)	51
PA			
Fish 1, Fish 2	0.249 (−0.046, 0.504)	−0.154 (−2.383, 2.076)	43
PA & Q2N			
PA, Q2N	0.301 (0.184, 0.409)	−1.783 (−3.564, −0.001)	52

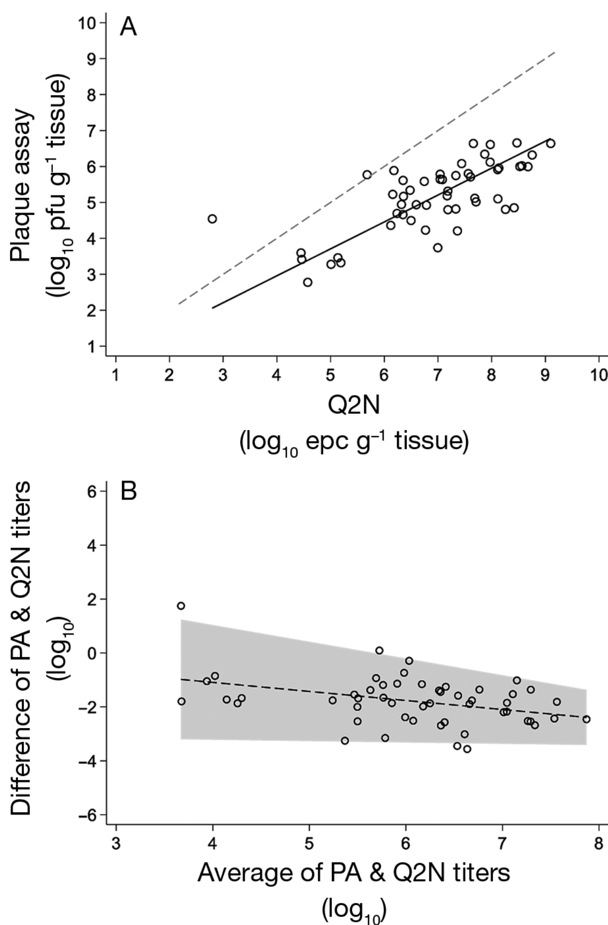


Fig. 5. Koi *Cyprinus carpio* L. exposed to spring viremia of carp virus. Pairwise analysis of Q2N and plaque assay (PA) average virus titer values for the same fish is presented in (A) the concordance correlation plot and (B) the Bland and Altman limits of agreement plot. In (A), the linear regression line of the test results is solid black, whereas the line of perfect concordance is dashed grey. In (B), $y = 0$ is the line of perfect agreement; the difference in virus titer values determined using the PA and Q2N test is represented by the dotted line where the grey zone represents the 95% confidence interval

approached their projected limits for sensitivity and repeatability and differed in their specificity. The linear range of the tests spanned at least 8 orders of magnitude, with ASe estimates for 100% LOD at 5 (Q2N) or 12.5 (Q1G) plasmid copies and >50% LOD at <3.13 plasmid copies. The higher sensitivity of the Q2N test reflects the relative abundance of nucleoprotein transcripts in infected material (Rose & Schubert 1987, Arakawa et al. 1990). The Q2N and Q1G tests yielded highly repeatable results both within and between runs. Thus the performance of the assays was consistent and reliable regardless of the virus load in the sample. The ASe of both tests is comparable to the sensitivity reported for other RT-qPCR tests designed to detect SVCV (Liu et al. 2008, Yue et al. 2008, Zhang et al. 2009, Misk et al. 2016, 2017, Shao et al. 2016). Since the ASe and repeatability of the Q2N and Q1G tests were similar, selection of which test to use can be guided by the ASp requirements of the testing.

The lower genetic diversity within the SVCV N gene (Teng et al. 2007) increases the likelihood of detecting emerging isolates with the trade-off of potential false positive results with closely related viruses. The ASp of the Q2N test was pan-specific for 28 SVCV isolates and exclusive of sympatric aquatic viruses except PFRV isolate GrCRV V76 (genus *Sprivivirus*). This means that the Q2N test was 100% inclusive detecting all of the carp sprivivirus isolates included in the study and 89% exclusive, since it did not detect other members of the family *Rhabdoviridae* tested or co-localizing viruses with the exception of GrCRV V76. In this case, despite sequence mismatches in 11 of the 55 nucleotides in the Q2N primer and probe binding regions, occasional amplification was observed. This finding indicates that the Q2N test may produce an SVCV false positive result if this isolate is present in a sample. The Cq value of 36.21 obtained with this isolate relative to the aver-

age Cq value of 17.89 observed with the other isolates reflects the 6 orders of magnitude lower binding efficiency of the Q2N primers and probe with the target sequence from isolate V76 (assuming 3 Cq is equivalent to 1 $\log_{10}$ order of magnitude). The threshold GrCRV V76 titer in the starting material above which false positive results may be observed is approximated from these results at 4.84 $\log_{10}$ pfu g^{-1}. This titer is 2 logs lower than the titer in fish that died in the first 35 d following immersion exposure (i.e. average titer 6.88 $\log_{10}$ pfu g^{-1}) and 2 logs higher than the average titer in convalescent fish sampled between 49 and 167 d following initial exposure (i.e. average titer 2.9 $\log_{10}$ pfu g^{-1}). Based on these values, false positive results are more likely to be observed with samples from fish with high virus titers (e.g. fish originating from a population experiencing a disease outbreak) and unlikely to occur with samples from fish with low titers (e.g. fish from asymptomatic, apparently healthy populations). A false positive result would be resolved through genotype and phylogenetic analyses that occur following detection of a virus. The pan-specific detection by Q2N of all SVCV isolates tested suggests that false negative results are most likely to arise when diagnosing fish that are infected with SVCV at virus titers below the LOD of 5 plasmid copies or with isolates such as RHV with primer/probe base mismatches that reduce assay efficiency.

The ASp of the Q1G test was narrower than that of Q2N given that Q1G was unable to detect 20120450, an isolate originally detected in common carp. The isolate was identified as belonging to carp sprivivirus genogroup Ia by the SVC OIE reference laboratory Shenzhen Exit & Entry Inspection and Quarantine Bureau (Shenzhen, China) (Emmenegger et al. 2018b). The false negative result obtained with Q1G is likely caused by 2 adjacent nucleotide mismatches located in the middle of the probe binding site. These results mean that the Q1G test was 96% inclusive, detecting all but 1 of the carp sprivivirus isolates included in the study and 100% exclusive since it did not detect other species within the genus *Sprivivirus*, family *Rhabdoviridae* or co-localizing viruses. The ASp of Q2N and Q1G is comparable to the exclusivity reported for other RT-qPCR tests designed to detect SVCV (Liu et al. 2008, Yue et al. 2008, Zhang et al. 2009, Misk et al. 2016, 2017, Shao et al. 2016).

Genetic changes observed in the Q1G target sequences represent a possible risk of future false negative results such as those obtained with isolate 20120450. Predicting genetic change in a viral genome as a result of evolution is difficult, but patterns have emerged from analyzing the dynamics of virus evolution (Salama et al. 2016). For example, immune selection (i.e. genetic mutation) occurs in genes encoding virus surface proteins in response to neutralizing antibodies produced by infected hosts. The latter are produced in response to SVCV infection in carp (Sulimanovic 1973, Hill et al. 1975, Fijan et al. 1977) and to vesiculoviruses, the closest relatives to spriviviruses (Björklund et al. 1996, Dietzgen et al. 2017), which show evidence of immune selection in the G protein sequence (Kelley et al. 1972, Vandepol et al. 1986, Munis et al. 2018). The region encoded by the Q1G target sequence (pre-cleavage amino acids 184–214) does not overlap with regions encoding neutralizing epitopes predicted for SVCV (Luo et al. 2014, Zhu et al. 2019) or vesicular stomatis virus (VSV) (Vandepol et al. 1986, Keil & Wagner 1989, Munis et al. 2018). Nevertheless, viral adaptation due to immune selection or other selection pressures acting in this region may pose a potential risk for future false negative results if nucleotide changes occur in the Q1G target sequence.

Structural and amino acid sequence comparisons of the VSV and SVCV glycoproteins show that the Q1G target sequence encodes a region that corresponds to the junction of the VSV G pleckstrin homology domain and the fusion domain (Roche et al. 2006, 2007). This region functions as a hinge which refolds during the pH-induced conformational change of the G protein from pre- to post-fusion forms (Roche et al. 2007). Sequence changes in this region would affect G protein structural rearrangement and membrane fusion and may either enhance or interfere with virus infection. Nucleotide sequence alignment analyses reveal that changes are occurring in this area of the SVCV genome. For example, maximum likelihood-based codon substitution analyses by Padhi & Verghese (2008, 2012) suggest that amino acids 216 and 217 of the SVCV glycoprotein are under positive selection. These residues do not include the region encoded by the Q1G target sequence but are immediately proximal and correspond to the 4-amino acid region (i.e. amino acids 215 to 218) identified by Zhang et al. (2009) as different in SVCV isolates from China. The variable amino acid residues at codon 216 are predicted to have little effect on functional divergence. Positive selection of codon 217 is specific to genogroup Ia. The occurrence of nucleotide changes within the Q1G target sequence in a genogroup Ia isolate (i.e. 20120450) is consistent with the observation of Padhi & Verghese (2012) that isolates within genogroup Ia are exhibiting a mean nucleotide substitution rate

across the glycoprotein gene of at least 5–7 times higher than their Id counterparts. Thus, a false negative result arising from a loss of target specificity is more likely to occur via a mutation event in Ia isolates. The likelihood of detecting changes in the ASp of Q1G is increased using a combination of tests like Q1G, Q2N and/or virus isolation and through periodic assessment of the ability of the test to detect emerging rhabdoviruses.

The Q2N test was used in parallel with a PA to detect virus and measure the relative virus abundance in koi following waterborne exposure to 1 of 8 isolates of SVCV from genogroups Ia to Id. SVCV was more abundant in fish sampled at earlier time points compared to asymptomatic convalescent fish that survived infection. Among the convalescent fish, the PA detected fewer positive fish compared to the Q2N test. This pattern was also observed by Dixon (2008), who found that SVCV could be isolated using cell culture from clinically infected fish but not from fish surviving infection. These results suggest that the ASe of cell culture-based tests for detection of SVCV is lower than that of the Q2N test. Thus, the more sensitive RT-qPCR Q2N or Q1G tests would be the preferred screening tests to use in surveillance programs that target apparently healthy populations of individuals in which pathogen load is likely to be low. In the latter case, confirmation of a positive test result achieved with either Q2N or Q1G may not be possible with the cell culture-based test. In the case of Q2N, this zone of uncertainty starts at virus loads below 6.5 $\log_{10}$ epc g^{-1} tissue (Cq >32.2; Fig. 6A). A notable exception to this trend was observed with SVCV isolate RHV. In this case, the PA was more sensitive than Q2N in detecting virus at the earlier time points as well as in convalescent fish (Fig. 6B). The lower efficiency of Q2N is evident in the Cq value of 24.73 obtained with this isolate versus the average Cq value of 17.56 observed with the other SVCV isolates. This shift represents a loss of least 2 orders of magnitude in the ASe of Q2N and is likely due to a single base mismatch (A to G) near the 3′ end of the reverse primer (Fig. S2). The reversal in the relative ASe of the 2 tests highlights the importance of designing diagnostic workflows that incorporate different techniques for detecting the virus.

With the exception of RHV, the SVCV isolates displayed the same virulence ranking (based on mortality) in koi in this study as determined by Emmenegger et al. (2018b): high, China 20040741, Fijan, NC2002; moderate, China 20120450, P4-7; low, China 20070165, 20100910. RHV grouped with the low rather than the moderate virulence isolates in our study. The lower cumulative mortality observed in our study may be related to the genotype and/or older age of the koi population (Shchelkunov & Shchelkunova 1989). Viremia due to SVCV is typically established following virus entry and dissemination from epithelial surfaces, particularly gill epithelia, to the blood and then internal organs including kidney and liver (Ahne 1977, 1978). Our finding of virus-exposed but not infected fish suggests that some fish are non-susceptible to SVCV. The MDD for the high virulence isolates was 17 (NC2002), 23 (20040741) or 20 (Fijan) dpe, suggesting that the peak infection phase of the exposure study occurred

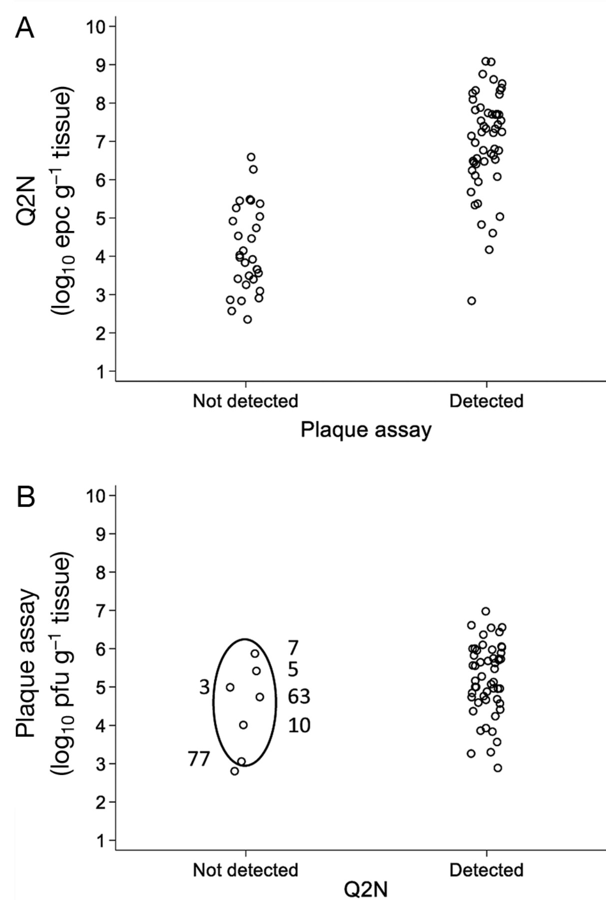


Fig. 6. Koi *Cyprinus carpio* L. exposed to spring viremia of carp virus (SVCV). Plaque assay (PA) and Q2N test results for koi collected between 1 and 167 d after exposure to SVCV. Virus titer is reported using (A) the Q2N test as $\log_{10}$ equivalent plasmid copies (epc) g^{-1} tissue and (B) as $\log_{10}$ plaque-forming units (pfu) g^{-1} tissue with the PA. Samples from isolate RHV-exposed fish that tested negative with the Q2N test but positive with the PA are circled in (B) and are labeled with the number of days after virus exposure when the sample was collected. A random jitter was added to expose the density of the data

within the first 20 d following exposure. Not all fish showed clinical signs of infection, but initial signs were observed 7–10 d following exposure to the high virulence isolates. We used experimental conditions similar to those described by Ahne (1978) which explains the comparable infection kinetics we observed for virus incubation (i.e. 7 dpe) and peak mortality (i.e. 20 dpe). Using this 13 d window of time to identify non-susceptible fish and evaluate their prevalence, we found that all of the fish that tested negative by both tests were from populations exposed to the low virulence isolates (9% prevalence) with the exception of 1 fish exposed to the moderate 20120450 isolate (2% prevalence). This inverse relationship was also observed from 35 to 167 dpe when the prevalence of non-susceptible fish increased with decreasing virus virulence in populations exposed to high (8%), moderate (15%) or low (30%) virulence isolates. A serology-based test could help distinguish non-susceptible fish that are resistant to infection from those that are resistant to disease.

Clustering the SVCV isolates based on their previously established virulence categories provided a framework for evaluating their infection kinetics. SVCV prevalence, titer, replicative rate and persistence correlated significantly with virulence (Fig. 4). The quantity of replicating virus was lower on average by 1.8 $\log_{10}$ relative to the quantity of nucleoprotein RNA, with peak production of both virus and RNA occurring in a similar time frame except for 2 high virulence isolates. In those cases, RNA production was high at earlier time points but peaked 7 to 10 d following peak production of replicating virus, suggesting that these isolates were able to produce more virus and then sustain high levels of nucleoprotein RNA production for longer. The analyses revealed that high virulence isolates were present in more fish, reached higher titers in shorter periods of time and persisted for longer in fish relative to moderate and low virulence isolates. These results suggest that SVCV virulence may be driven by multiple viral fitness traits including virus replication and virus persistence, in which the latter implies continued virus replication (as described by Kane & Golovkina 2010). These biological traits are similar to those of VSV whose hallmark feature is its rapid replication (Hastie et al. 2013). The slower replication rate of the less virulent isolates in our study may have facilitated the ability of the host immune system to resist or limit infection and clear the virus. The decline in virus titer over time for isolates from all virulence categories suggests that if SVCV persistence requires continuous virus replication, then it likely occurs at a limited rate and in a select group of cells as shown for other RNA viruses of fish (Dahle & Jørgensen 2019). Persistence of highly virulent SVCV in survivors may lead to chronic disease or cycles of reactivated disease. Cycling of the virus in a temperature-dependent manner between persistent and acute infection stages in spawning fish could create a virus reservoir as well as a transmission pathway that maintains the virus within a population. Without a test capable of detecting low virus loads in survivors, movement of these fish represents a potential risk for introducing SVCV into a naïve population of susceptible fish or aquatic organisms. The RT-qPCR tests Q2N and Q1G reduce this risk and provide a method for evaluating the molecular mechanism by which SVCV establishes a persistent infection.

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