UNITED STATES OF AMERICA – COMMENTS

Annex 26. Item 9.2.2. – Chapter 2.3.9. 'Infection with spring viraemia of carp virus'

CHAPTER 2.3.9

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

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4. Diagnostic methods

The methods currently available for pathogen detection that can be used in i) surveillance of apparently healthy animals, ii) presumptive diagnosis in clinically affected animals and iii) confirmatory diagnostic purposes are listed in Table 4.1. by animal life stage.

Ratings for purposes of use. For each recommended assay a qualitative rating for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

+++ =	Methods are most suitable with desirable performance and operational characteristics.
++ =	Methods are suitable with acceptable performance and operational characteristics under most circumstances.
+ =	Methods are suitable, but performance or operational characteristics may limit application under some circumstances.
Shaded boxes =	Not appropriate for this purpose.

Validation stage. The validation stage corresponds to the assay development and validation pathway in chapter 1.1.2. The validation stage is specific to each purpose of use. Where available, information on the diagnostic performance of recommended assays is provided in Section 6.3.

WOAH Reference Laboratories welcome feedback on diagnostic performance of recommended assays, in particular PCR methods. Of particular interest are any factors affecting expected assay sensitivity (e.g. tissue components inhibiting amplification) or expected specificity (e.g. failure to detect particular genotypes, detection of homologous sequences within the host genome). These issues should be communicated to the WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts												
Histopathology												
Cytopathology												
Cell culture		++	++	1		++	++	1		++	++	1
Real-time RT-PCR		+++	+++	1		+++	+++	3		+++	+++	3
Conventional RT-PCR		~~++~~	~~++~~	~~1~~		++	++	1		++	++	1
Amplicon sequencing										+++	+++	1
In-situ hybridisation												
Immunohistochemistry						++	++	1				
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA						++	++	1				
IFAT						++	++	1				

LV = level of validation, refers to the stage of validation in the WOAH Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay.

[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6).

[2]Early and juvenile life stages have been defined in Section 2.2.3.

Shading indicates the test is inappropriate or should not be used for this purpose.

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section B.2.5 *Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis* of Chapter 2.3.0 *General information* (diseases of fish). Each sample should be tested in duplicate.

Real-time RT-PCR assays have been developed to detect and confirm SVCV (Clouthier *et al.,* 2021; Misk *et al.,* 2017; Rice, 2022; Shoa *et al.,* 2016; Yue *et al.*, 2008; Zhang *et al.,* 2009), however, sensitivity and specificity might not be acceptable for all genotypes of SVCV. Based on the genome sequences of SVCV strains of variant genotypes, an optimised real-time RT-PCR has been validated and is recommended (Rice, 2023; Zhu *et al.,* 2026).

A conventional nested RT-PCR has been widely used for the detection and confirmation on cell culture and fish tissue extracts, and the sequence of the nested-PCR amplified products can be used for genotyping phylogenetic analyses (Stone *et al.*,2003). Additional conventional RT-PCR assays are available for the detection and confirmation of SVCV infections (Koutna *et al.,* 2003; Shimahara *et al.,* 2016), while only the conventional RT-PCR assay developed by Shimahara *et al.* (2016) was sufficiently validated to identify the full range of SVCV genotypes on cell culture.

United States	**Category:** change **Proposed amended texts (or precise suggested deletion):** Please see the proposed changes in blue – "Real-time RT-PCR assays have been developed to detect and confirm SVCV (Clouthier *et al.,* 2021; Misk *et al.,* 2017; Rice, 2022~~2~~3;…" "…Additional conventional RT-PCR assays are available for the detection and confirmation of SVCV infections (Koutna *et al.*, 2003; Shimahara *et al.*, 2016), while only the conventional RT-PCR assay developed by Shimahara *et al.* (2016) was sufficiently validated ~~to~~for the purpose of genotyping SVCV isolates~~identify the full range of SVCV genotypes on cell culture~~." **Rationale:** The Rice reference mentioned was published in 2023, not 2022. Additionally, we suggest alternative wording regarding SVC genotyping to improve scientific accuracy and clarity.

4.4.1. Real-time RT-PCR

~~The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control if available and validated.~~

~~Real-time RT-PCR assays are available to detect and confirm SVCV (Yue *et al.*, 2008; Zhang *et al.*, 2009), however, they are not currently recommended as they have not been sufficiently validated.~~

Table 4.4.1.1. Real-time RT-PCR: primer/probe sequences and cycling parameters

Pathogen/target gene	Primer/probe (5'-3')	Concentration	Cycling parameters[(a)]
Method 1: (Rice 2023; Zhu *et al.,* 2026; GenBank Accession No.: U18101)			
SVCV/RNA polymerase gene	SVCV Cefas AR-F: CAA-TGG-GAA-TGA-GTG-CTT-CT SVCV Cefas AR-R: TGA-TTT-GGT-TTT-GAA-TTG-TRT-C SVCV Cefas AR-P: AGA-CAG-AGG-TRA-AAG-GAC-ATC-TGA-T	0.4 µm of each primer 0.2 µm of probe	Reverse transcription step at 45°C/15 min 40 cycles of 95°C/15 sec and 58°C/60 sec

[(a)] A denaturation step prior to cycling has not been included.

4.4.2. Conventional RT-PCR

~~Positive and negative controls should be run with each stage of the assays: extraction, RT-PCR and second round PCR. Due to the sensitive nature of PCR-based assays it is highly recommended that master mix, template addition and PCR amplification occur in designated hoods or spatially separated areas.~~

~~Nested reverse-transcription polymerase chain reaction (RT-PCR) (confirmation of virus identity from cell culture isolation or directly from fish tissue extracts)~~

~~The genome of SVCV consists of a single strand of RNA of approximately 11 kb, with negative polarity. Amplification of a 714 bp fragment of SVCV cDNA is performed using primers derived from sequences of the region coding for the glycoprotein gene: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY*-3' SVCV R2), using a modification of the method of Stone *et al.* (2003).~~

~~i) Total RNA is extracted from 100 µl of supernatant from cell cultures exhibiting CPE or 50 µl of fish tissue extract and dissolved in 40 µl molecular biology grade DNase- and RNase-free water.~~

~~A number of total RNA extraction kits are available commercially that will produce high quality RNA suitable for RT-PCR.~~

~~ii) For cDNA synthesis, a reverse transcription reaction is performed at 37°C for 1 hour in a 20 µl volume consisting of 1 × M-MLV RT reaction buffer (50 mM Tris, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$) containing 1 mM dNTP, 100 pmol SVCV R2 primer, 20 units M-MLV reverse transcriptase (Promega, Southampton, UK) or an equivalent reverse transcriptase system and 1/10 of the total RNA extracted above.~~

~~iii) RT-PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R2 and SVCV F1 primers, 1.25 units of Taq DNA polymerase, and 2.5 µl reverse transcription reaction mix. The reaction mix is subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (714 bp) is analysed by agarose gel electrophoresis.~~

~~iv) If the CPE in culture is not extensive it is possible that a visible product will not be generated using a single round of amplification. To avoid such problems, use the semi-nested assay using primers: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR* R*TC-3' (SVCV F1) and 5' CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3' (SVC R4) according to Stone *et al.* (2003).~~

~~v) The second round of PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R4 and SVCV F1 primers, 1.25 units Taq DNA polymerase, and 2.5 µl of the first round product. The reaction mix is subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (606 bp) is analysed by agarose gel electrophoresis.~~

~~vi) All amplified products are confirmed as SVCV in origin by sequencing, and the SVCV subtype (Ia-Id) is identified using a BLAST search (http://www.ebi.ac.uk/blastall/index.html) or by phylogenetic analysis using the SVCV sequences available in public sequence databases. Phylogenetic analysis is undertaken using a 426 bp region corresponding to nucleotides 429–855 of the glycoprotein gene.~~

~~vii) In cases where the CPE is extensive and the virus replicates to a high titre, or where a semi-nested RT-PCR assay was used, sufficient PCR amplicon will be available for direct sequencing. Where the amplified product is weak it is recommended that the product be inserted into an appropriate sequencing vector (e.g. pGEM-T, pCR® 4-TOPO®) prior to undertaking the sequencing. At least two independent amplification and sequencing events should be undertaken to eliminate potential sequence errors introduced by the Taq polymerase.~~

~~*Reverse-transcription polymerase chain reaction* (*RT-PCR*) (*confirmation of virus identity*)~~

~~Additional conventional RT-PCR assays are available to detect and confirm SVCV infections (Koutna *et al.*, 2003; Shimahara *et al.*, 2016). A generic primer set based on the polymerase gene also identifies viruses from both the Sprivivirus and Perhabdovirus genera and can be used to screen a virus culture (Ruane *et al.*, 2014). With the exception of the conventional RT-PCR assay developed by Shimahara *et al.* (2016) the other assays were not sufficiently validated against representatives from each of the recognised SVCV genogroups and they may fail to detect the full range of SVCV genotypes.~~

~~A summary of the Shimahara *et al.* (2016) RT-PCR method follows. Amplification of a 369 bp fragment of SVCV glycoprotein gene is performed using primers as follows: SVCV-G1: 5' TGA-AGA-YTG-TGT-CAA-TCA-AGTC-3'~~

~~and SVCV-G2: 5'-GCG-ART-GCA-GAG-AAA-AAG-TG-3'. Preparation of RNA template is the same as nested RT-PCR above. Reverse transcription of SVCV RNA and amplification of cDNA are carried out using SuperScript III one-step RT-PCR with PlatinumR Taq (Invitrogen) according to the manufacturer's instructions. The RT-PCR reaction mixture contained 10 pmol of each primer, 12.5 µl of 2× reaction mix, 1 µl of SuperScript III RT/Platinum Taq Mix and 2.5 µl template. After reverse transcription at 50°C for 30 minutes and 94°C for 2 minutes, 40 amplification cycles of 94°C for 15 seconds, 56°C for 30 seconds and 68°C for 1 minute followed by a final extension step at 68°C for 7 minutes is performed. All amplified products are confirmed as SVCV in origin by sequencing.~~

~~Primer~~	~~Sequence (5'-3')~~	~~Product size (bp)~~	~~Final primer concentration~~	~~Reference~~
~~SVCV F1~~	~~TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC~~	~~714~~	~~0.15µM~~	~~Stone *et al.*, 2003~~
~~SVCV R2~~	~~AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY*~~		~~0.05µM~~	
~~SVCV F1~~	~~TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC~~	~~606~~	~~0.05µM~~	
~~SVC R4~~	~~CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*~~		~~0.05µM~~	
~~SVCV-G1~~	~~TGA-AGA-YTG-TGT-CAA-TCA-AGT-C~~	~~369~~	~~0.05µM~~	~~Shimahara *et al.*, 2016~~
~~SVCV-G2~~	~~GCG-ART-GCA-GAG-AAA-AAG-TG~~		~~0.05µM~~	

Table 4.4.2.1. Conventional RT-PCR: primer sequence and cycling parameters

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters[a]
Method 1: Stone *et al.,* 2003; GenBank Accession No.: U18101; amplicon size: 714/606 bp			
SVCV/ glycoprotein gene	SVCV R2: AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY* SVCV F1: TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC SVCV F1: TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC SVC R4: CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*	Reverse transcription:100 pmol SVCV R2 For primary amplification: 50 pmol each of R2 and F1 For nested amplification: 50 pmol each of R4 and F1	Reverse transcription: 37°C/60 min For primary amplification: 35 cycles of: 95°C/60sec, 55°C/60 sec and 72°C/60 sec For nested amplification: 35 cycles of: 95°C/60 sec, 55°C/60 sec and 72°C/60 sec
Method 1 (Stone et al.,2003; GenBank Accession No.U18101, amplicon size: 714/606 bp)			
SVCV/ glycoprotein gene	SVCV-G1: TGA-AGA-YTG-TGT-CAA-TCA-AGT-C SVCV-G2: GCG-ART-GCA-GAG-AAA-AAG-TG*	10 pmol	Reverse transcription: 50°C/30 min 40 cycles of: 95°C/15 sec, 55°C/60 sec and 68°C/60 sec

[a]A denaturation step prior to cycling has not been included.

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5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

~~The method for surveillance of apparently healthy populations for declaration of freedom from infection with SVCV is inoculation of cell culture with tissue homogenates (as described in Section 4.3). Cell culture is considered the most suitable method despite the lack of validation data for diagnostic methods for SVCV.~~

Virus isolation in cell culture or real-time RT-PCR are the recommended tests for surveillance to demonstrate freedom from infection with SVCV.

6. Corroborative diagnostic criteria

This Section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1) or in the presence (Section 6.2) of clinical signs but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory.

6.1. Apparently healthy animals or animals of unknown health status[1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link to an infected population. Geographical proximity to, or movement of animals or animal products or equipment from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with SVCV shall be suspected if at least one of the following criteria is met:

i) Positive result by conventional RT-PCR

ii) Positive result by real-time RT-PCR

iii) SVCV-typical CPE. in cell culture

United States	**Category:** editorial **Proposed amended texts (or precise suggested deletion):** Please see the proposed edit in blue – “SVCV-typical CPE. in cell culture" **Rationale:** Grammatical edit.

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with SVCV is considered to be confirmed if at least one of the following ~~criterion~~ criteria is met:

i) Virus isolation with SVCV-typical CPE in cell culture and virus identification by conventional RT-PCR followed by ~~virus identification by conventional RT-PCR test and~~ amplicon sequencing.

ii) Virus isolation with SVCV-typical CPE in cell culture and virus identification by real-time RT-PCR

iii) Positive result by real-time RT-PCR in tissue preparations and positive result by conventional RT-PCR followed by sequencing of the amplicon

6.2. Clinically affected animals

Clinical signs are not pathognomonic for infection with SVCV; however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with SVCV shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality

ii) Positive result by conventional RT-PCR

iii) Positive result by real-time RT-PCR

~~ii~~ iv) Positive result by antigen ELISA

~~i~~ v) Positive result by IFAT

1 For example transboundary commodities.

vi) Positive result by immunohistochemistry

vii) SVCV-typical CPE in cell culture

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with SVCV is considered to be confirmed if at least one of the following ~~criterion~~ criteria is met:

i) Virus isolation with SVCV-typical CPE in cell culture ~~followed by~~ and virus identification by conventional RT-PCR ~~test and~~ followed by amplicon sequencing

ii) Virus isolation with SVCV-typical CPE in cell culture and virus identification by real-time RT-PCR

iii) Positive result by real-time RT-PCR in tissue preparations and positive result by conventional RT-PCR followed by sequencing of the amplicon

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with SVCV is provided in Tables 6.3.1. and 6.3.2 (no data are currently available ~~for either~~). This information can be used for the design of surveys for infection with SVCV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level 2 of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

6.3.1. For presumptive diagnosis of clinically affected animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Real-time RT-PCR	Clinical diagnosis	Fish collected in surveillance programmes and experimentally infected fish	Cell culture	Common carp and koi	100 (249)	100 (15)	Conventional RT-PCR with sequencing	Zhu *et al.*, 2026
Real-time RT-PCR	Clinical diagnosis	Fish collected in surveillance programmes and experimentally infected fish	Gill, kidney and spleen	Common carp and koi	96.6 (189)	100 (15)	Cell culture and conventional RT-PCR with sequencing	Zhu *et al.*, 2026

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study.

United States	**Category:** change **Proposed amended texts (or precise suggested deletion):** Please see the proposed changes in blue – "~~Gill,~~ kidney and spleen" "~~Cell culture~~Virus isolation and identification by conventional RT-PCR with amplicon sequencing" **Rationale:** Gill should not be included under "Tissue or sample types" because while the assay provides a practical tool for laboratory detection, its performance in broader surveillance and clinical diagnostics requires additional validation as stated in https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2026.1805875/full. Additionally, we suggest alternative wording to improve scientific accuracy and clarity.

6.3.2. For surveillance of apparently healthy animals (no data currently available)

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study.

7. References

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NB: There are WOAH Reference Laboratories for spring viraemia of carp (~~see Table at the end of this *Aquatic Manual* or~~ please consult the WOAH web site: http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the WOAH Reference Laboratories for any further information on spring viraemia of carp

NB: First adopted in 1995 as spring viraemia of carp. Most recent updates adopted in 2023.