CHAPTER 2.3.9

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

1. Scope

Infection with spring viraemia of carp virus means infection with the pathogenic agent Carp sprivivirus (commonly known as spring viraemia of carp virus [SVCV]), of the Genus *Sprivivirus* and the Family *Rhabdoviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

The virus genome is a non-segmented, negative-sense, single strand of RNA. The genome contains 11,019 nucleotides encoding five proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G) and an RNA-dependent, RNA polymerase (L). The genome does not contain a non-virion (NV) gene between the G and L genes as is found in fish rhabdoviruses of the genus *Novirhabdovirus* (Ahne *et al*., 2002). The type strain of SVCV is available from the American Type Culture Collection (ATCC VR-1390). Two complete genome sequences of the type strain have been submitted to Genbank (Genbank accession U18101 by Bjorklund *et al*. [1996] and Genbank accession AJ318079 by Hoffmann *et al*. [2002]). The complete genome sequence of isolates from China (People's Rep. of) has also been deposited in Genbank (Genbank accession DQ097384 by Teng *et al*. [2007] and Genbank accession EU177782 by Zhang *et al*. [2009]).

Stone *et al*. (2003) used sequence analysis of a 550 nucleotide region of the G-gene to compare 36 isolates from different fish species and geographical locations that were previously identified by serology as SVCV or pike fry rhabdovirus (PFRV). The analysis showed that the isolates could be separated into four distinct genogroups and that all of the SVCV isolates could be assigned to genogroup I, sharing <61% nucleotide identity with viruses in the other three genogroups. Re-analysis of the sequence data generated for viruses assigned to Genogroup I identified four subgroups (Ia–d). Those viruses originating in Asia were assigned to Subgroup Ia, those from Moldova, the Ukraine and Russia to Subgroups Ib and Ic, and those from the UK to Subgroup Id.

2.1.2. Survival and stability in processed or stored samples

There are limited published data on the stability of the pathogen in host tissues. There is also limited information on the stability of the virus in the tissues after death of a diseased animal. Detection of SVCV in the tissues of recently dead animals by either RT-PCR or culture may be possible, and therefore, dead fish may be taken for analysis if moribund fish are not available.

The virus can be stored for several months when frozen in medium containing 2–5% serum. The virus is most stable at lower temperatures, with little loss of titre when stored at –20°C for 1 month, or at –30 or –74°C for 6 months (Ahne, 1976; de Kinkelin & Le Berre, 1974). The virus remains stable over four freeze (–30°C)–thaw cycles in medium containing 2% serum (de Kinkelin & Le Berre, 1974).

2.1.3. Survival and stability outside the host

The virus can remain infectious outside the host for 5 weeks in river water at 10°C and for more than 6 weeks in pond mud at 4°C, reducing to 4 days in pond mud at 10°C (Ahne, 1976).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with SVCV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are:

Family	Scientific name	Common name
Cyprinidae	*Abramis brama*	bream
	Aristichthys nobilis	bighead carp
	Carassius auratus	goldfish
	Ctenopharyngodon idella	grass carp
	Cyprinus carpio	common carp (all varieties and subspecies)
	Danio rerio	zebrafish
	Notemigonus crysoleucas	golden shiner
	Pimephales promelas	fathead minnow
	Percocypris pingi	Jinsha barbel carp
	Rutilus kutum	Caspian white fish
	Rutilus rutilus	roach
Siluridae	*Silurus glanis*	wels catfish

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* are: crucian carp (*Carassius carassius*), pike (*Esox lucius*), firebelly newt (*Cynops orientalis*), silver carp (*Hypophthalmichthys molitrix*), and yellow perch (*Perca flavescens*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated:

Family	Scientific name	Common name
Catostomidae	*Catostomus commersonii*	white sucker
Cichlidae	*Oreochromis niloticus*	Nile tilapia
Cyprinidae	*Notropis atherinoides*	emerald shiner
	Cirrhinus mrigala	mrigal carp
	Labeo rohita	rohu
	Tinca tinca	tench
Penaeidae	*Litopenaeus vannamei*	Pacific white shrimp
Salmonidae	*Oncorhynchus tshawytscha*	chinook salmon
	Oncorhynchus nerka	sockeye salmon
	Oncorhynchus mykiss	rainbow trout

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

Common carp varieties are the principal hosts for SVCV and are considered to be most likely to be infected with SVCV followed by other carp species (including hybrids), other susceptible cyprinid species and finally non-cyprinid fish species.

Generally, young fish up to one-year old are most likely to demonstrate clinical signs of disease, but all age groups can be affected. Moreover, there is a high variability in the degree of susceptibility to infection with SVCV among individuals of the same fish species. Apart from the physiological state of the fish, the role of which is poorly understood, age or the age-related status of innate immunity appears to be extremely important to the manifestation of clinical disease: younger fish are more likely to show signs of overt disease.

Fish that have separated from the shoal and found at the water inlet or sides of a pond are more likely to be infected.

For the purposes of Table 4.1 carp larvae and fry (e.g. up to approximately 1 g in weight) may be considered early life stages, carp may be considered juveniles (i.e. fingerlings and grower fish) up to 250 g, and adults are above 250 g.

2.2.4. Distribution of the pathogen in the host

SVCV appears to enter via the gills and then spread to the kidney, liver, heart, spleen and alimentary tract. During disease outbreaks high titres of virus occur in the liver and kidney of infected fish, but much lower titres occur in the spleen, gills and brain (Dixon, 2008). The virus has been detected in ovarian fluid (Bekesi & Csontos, 1985).

2.2.5. Aquatic animal reservoirs of infection

Liu *et al*. (2004) isolated SVCV in China (People's Rep. of) from common and koi carp exhibiting no external or internal signs of disease, and similarly, the virus was isolated from apparently healthy wild carp in Canada (Garver *et al*., 2007). Thus fish with long-term subclinical infections may act as reservoirs of infection.

2.2.6. Vectors

The parasitic invertebrates *Argulus foliaceus* (Crustacea, Branchiura) and *Piscicola geometra* (Annelida, Hirudinea) have been demonstrated to transfer SVCV from diseased to healthy fish under experimental conditions and the virus has been isolated from A. foliaceus removed from infected carp (Ahne *et al*., 2002; Dixon, 2008). It has been demonstrated experimentally that virus can be isolated from fish tissues regurgitated by herons (*Ardea cinerea*) 120 minutes after being fed with SVCV-infected carp, suggesting a potential route for SVCV transmission, but is not known whether such transmission has occurred in nature (Peters & Neukirch, 1986).

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

A noticeable increase in mortality will occur in the population during an outbreak of infection with SVCV. Disease patterns are influenced by water temperature, age and condition of the fish, population density and stress factors. The immune status of the fish is also an important factor with both nonspecific (e.g. interferon) and specific immunity (serum antibodies, cellular immunity) having important roles. Poor physiological condition of over-wintered fish may be a contributory factor to the onset of clinical disease in infected animals. In European aquaculture, losses can be up to 70% in young carp (Ahne *et al*., 2002), but are usually from 1 to 40%.

In one survey from Serbia, the virus was isolated by culture in samples collected from 12 of the 38 hatcheries screened over the 10-year period (1992–2002) (Svetlana *et al*., 2004). The virus occurred sporadically in different ponds on one site, and sporadically from year to year at different sites (Svetlana *et al*., 2004). In another study, 18 of 30 tissue pools (five fish/pool) of wild, clinically health, common carp sampled in Canada in 2006 were positive for SVCV by culture (Garver *et al*., 2007). This observation suggests that SVCV infection may be clinically inapparent (Fijan, 1999).

2.3.2. Clinical signs, including behavioural changes

Fish can become lethargic, separate from the shoal and gather at the water inlet or sides of a pond and some may experience loss of equilibrium. Clinical signs of infection with SVCV are nonspecific and not all fish will exhibit all of the signs. Two of the most obvious and consistent features are abdominal distension and haemorrhages, which may be pale and occur on the skin, fin bases, eyes and gills. The skin may darken and exophthalmia is often observed. The vent may be swollen, inflamed and trail mucoid casts. There may be no clinical signs in cases with a sudden onset of mortality.

2.3.3 Gross pathology

There are no pathognomonic gross lesions. Lesions may be absent in cases of sudden mortality. Gross pathologies are mainly documented for common carp and may include excess ascitic fluid in the abdominal cavity, usually containing blood, degeneration of the gill lamellae and inflammation of the intestine, which contains mucous instead of food. Oedema and haemorrhage of the visceral organs is commonly observed (the spleen is often enlarged), and organs adhere to each other and to the peritoneum. Focal haemorrhages may be seen in the muscle and fat tissue, as well as in the swim bladder (see Dixon, 2008). However, petechial haemorrhages are infrequent in cases caused by Asian strains of SVCV (Dikkeboom *et al*., 2004).

2.3.4. Modes of transmission and life cycle

The transmission of SVCV is horizontal (Fijan, 1988). Horizontal transmission may be direct, or via water, fomites or vectors (Section 2.2.7) (Fijan, 1988). The virus appears to enter the host via the gills. A viraemia follows and the virus rapidly spreads to the liver, kidney, spleen and alimentary tract. The virus can be detected in faeces and is also shed into the water via faeces and urine (Ahne, 1982).

Vertical and 'egg-associated' transmission cannot be ruled out following one report of isolation of SVCV from carp ovarian fluid, although there have been no further reports (Bekesi & Csontos, 1985).

2.3.5. Environmental factors

Disease outbreaks in carp generally occur between 11 and 17°C. They rarely occur below 10°C, and mortalities, particularly in older fish, decline as the temperature exceeds 22°C (Fijan, 1988). However, the virus was isolated from apparently healthy fish from a lake in Canada that had been sampled over a 13-day period during which the water temperature varied between 24.2°C and 27.3°C (Garver *et al*, 2007). These fish may have been more susceptible to infection as they were penned and detection was during spawning. Secondary and concomitant bacterial or parasitic infections can affect the mortality rate and the appearance of clinical signs. In carp, the disease is often observed during spring (hence the common name for the disease), particularly in countries having cold winters. It is believed that the poor condition of the over-wintered fish may be a contributory factor in the occurrence of clinical disease. Clinical disease can occur in fish in quarantine following the stress of transportation, even though there has been no evidence of infection prior to transportation.

2.3.6. Geographical distribution

For a long time, the geographical range of SVCV was limited to countries of the European continent that experience low water temperatures during winter. The disease has been recorded from most European countries. However, in 1998, the disease was recorded in South America and in 2002 in North America. The virus was first detected in Asia in 2004.

See WOAH WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

A safe and effective vaccine is not currently available; however, the efficacy of an experimental DNA vaccine has been investigated (Emmenegger & Kurath, 2008).

2.4.2. Chemotherapy including blocking agents

Methisoprinol inhibits the replication of SVCV *in vitro*, but has not been tested under carp culture conditions (Siwicki *et al*., 2002).

2.4.3. Immunostimulation

Injection into carp of single-stranded and double-stranded RNA (which is an interferon inducer) protected carp for longer than 3 weeks, but the treatment is not effective by bath administration (Alikin *et al*., 1996).

2.4.4. Breeding resistant strains

The "Krasnodar" strain of common carp has been bred for increased resistance to SVCV (Kirpichnikov *et al*., 1993).

2.4.5. Inactivation methods

The virus is inactivated at 56°C for 30 minutes, at pH 12 for 10 minutes and pH 3 for 2 hours (Ahne, 1986). Oxidising agents, sodium dodecyl sulphate, non-ionic detergents and lipid solvents are all effective for inactivation of SVCV. The following disinfectants inactivate the virus: 3% formalin for 5 minutes, 2% sodium hydroxide for 10 minutes, 540 mg litre^{-1} chlorine for 20 minutes, 200–250 ppm (parts per million) iodine compounds for 30 minutes, 100 ppm benzalkonium chloride for 20 minutes, 350 ppm alkyltoluene for 20 minutes, 100 ppm chlorhexidine gluconate for 20 minutes and 200 ppm cresol for 20 minutes (Ahne, 1982; Ahne & Held, 1980; Kiryu *et al*., 2007).

2.4.6. Disinfection of eggs and larvae

Eggs can be disinfected by iodophor treatment (Ahne & Held, 1980).

2.4.7. General husbandry

Methods to control infection with SVCV rely on avoiding exposure to the virus coupled with good hygiene practices. This is feasible on small farms supplied by spring or borehole water and a secure system to prevent fish entering the farm via the discharge water. Reducing fish stocking density during winter and early spring will reduce the spread of the virus. In rearing facilities with a controlled environment, elevation of water temperature above 19–20°C may stop or prevent outbreaks of infection with SVCV.

3. Specimen selection, sample collection, transportation and handling

This Section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples which are most likely to be infected.

3.1. Selection of populations and individual specimens

Clinical inspections should be carried out during a period when the water temperature is between 11°C and 17°C. All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. Particular attention should be paid to the water outlet area where weak fish tend to accumulate due to the water current.

For the purposes of disease surveillance, fish to be sampled are selected as follows:

i) Common carp or strains such as koi or ghost (koi × common) carp are preferentially selected, followed by carp hybrids (e.g. common carp × crucian carp), then other cyprinid species such as goldfish, grass carp, bighead carp, bream and roach. Susceptible species should be sampled proportionally, or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown disease status).

ii) If more than one water source is used for fish production, fish from the highest risk water source should be targeted. If all water sources are of equal risk, all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish should be selected. If such fish are not present, the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with SVCV should be collected. Ideally fish should be collected while alive, however, recently dead fish can also be selected for diagnostic testing. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time. There may be no clinical signs or gross pathognomonic lesions in cases of sudden mortality.

3.2. Selection of organs or tissues

Kidney, spleen, gill and encephalon should be selected from apparently healthy fish.

For clinically affected fish: whole fry (body length ≤ 4 cm), entire viscera including kidney and brain (> 4 cm body length ≤ 6 cm) or, for larger fish, liver, kidney, spleen and encephalon should be selected.

3.3. Samples or tissues not suitable for pathogen detection

Decomposed clinical samples and seminal fluid samples are not suitable. While the virus has been isolated at low frequency from ovarian fluids, the suitability of these tissues for detection of SVCV has not been substantiated (Bekesi & Csontos, 1985).

3.4. Non-lethal sampling

Serological assays for antibodies can be undertaken on blood samples and can indicate possible exposure to SVCV, however, serology is not a suitable test for making a suspect diagnosis.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0. *General information* (diseases of fish).

3.5.1. Samples for pathogen isolation

For recommendations on transporting samples for virus isolation to the laboratory, see Section B.2.4 of Chapter 2.3.0 *General information* (diseases of fish).

3.5.2. Preservation of samples for molecular detection

The material collected for virus culture is generally used for the molecular diagnostic assays, but additional tissue samples for RT-PCR can be preserved in commercially available RNA preservation solutions according to the manufacturers' recommendations, or, alternatively, samples can be preserved in 80–90% (v/v) analytical grade (absolute) ethanol at the recommended ratio of ethanol to tissue of 10:1.

3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Standard methods for histopathology can be found in Chapter 2.3.0 *General information* (diseases of fish).

3.5.4. Samples for other tests

Tubes for the separation of serum are available commercially. After collection, the blood is allowed to clot by leaving it undisturbed at room temperature. This usually takes 15–30 minutes. Serum is clarified by centrifuging at 1000–2000 ***g*** for 10 minutes at 4–8°C.

3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where supporting data on diagnostic sensitivity and diagnostic specificity are available. However, smaller life stages (e.g. fry) can be pooled to provide a minimum amount of material for testing.

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 PCR												
Conventional 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.

4.1. Wet mounts

Not applicable.

4.2. Histopathology and cytopathology

Histopathological changes can be observed in all major organs. In the liver, blood vessels show oedematous perivasculitis progressing to necrosis. Liver parenchyma shows hyperaemia with multiple focal necrosis and degeneration. The heart shows pericarditis and infiltration of the myocardium progressing to focal degeneration and necrosis. The spleen shows hyperaemia with hyperplasia of the reticuloendothelium and enlarged melanomacrophage centres, and the pancreas is inflamed with multifocal necrosis. In the kidney, damage is seen to excretory and haematopoietic tissue. Renal tubules are clogged with casts and the cells undergo hyaline degeneration and vacuolation. The intestine shows perivascular inflammation, desquamation of the epithelium and atrophy of the villi. The peritoneum is inflamed, and lymph vessels are filled with detritus and macrophages. In the swim bladder, the epithelial lamina changes from a monolayer to a discontinuous multi-layer and vessels in the submucosa are dilated with nearby lymphocyte infiltration.

As the histopathological presentation is not specific for the disease, and not all fish will exhibit each feature (Misk *et al*., 2016), microscopic methods by themselves are not recommended for diagnosis of SVCV. They may, however, provide supporting evidence, particularly, when immunohistochemistry or nucleic acid based *in-situ* hybridisation methods are used (see the relevant Sections below).

4.3. Cell culture for isolation

4.3.1. Cell lines

The recommended cell lines for SVCV detection are EPC, FHM or GCO. Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

EPC, FHM and GCO cells are grown at 20–30°C in suitable medium, e.g. Eagle's minimal essential medium (MEM or modifications thereof) with a supplement of 10% fetal bovine serum (FBS) and antibiotics in standard concentrations. When the cells are cultivated in closed vials, it is recommended to buffer the medium with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris-HCl (23 mM) and Na-bicarbonate (6 mM). The pH must be 7.6 ± 0.2. Cell culture plates should be seeded 4–48 hours and not 100% confluent prior to inoculation. 15–30 minutes prior to sample inoculation, cells should be pre-treated with 7% (w/v) PEG-20,000 solution (10–15 µl/cm^2) (Batts & Winton, 1989; Wang *et al*., 2016).

4.3.2. Sample preparation and inoculation

Virus isolation: Use the procedure described in Section 2.2.2 of Chapter 2.3.0 *General information* (diseases of fish).

Inoculation of cell monolayers: make two serial tenfold dilutions of the 1/10 organ homogenate supernatants in cell culture medium (i.e. the homogenate supernatants will be 1/100 and 1/1000 dilutions of the original organ material) and transfer an appropriate volume of each of these two dilutions on to 24-hour-old cell monolayers drained of their culture medium. Alternatively, make a single tenfold dilution of the 1/10 organ homogenate (i.e. a 1/100 dilution of the original organ material) and add an appropriate volume of both the 1/10 and 1/100 dilutions directly to undrained 24 hour-old cell monolayers, to effect 1/100 and 1/1000 final dilutions of the organ homogenate. Should toxicity of the sample be a problem, make two serial tenfold dilutions of the 1/10 organ homogenate supernatants in cell culture medium as described above and inoculate at least 2 cm^2 of drained cell monolayer with 100 µl of each dilution. Allow to adsorb for 0.5–1 hour at 10–15°C, withdraw the inoculum and add cell culture medium buffered at pH 7.6 and supplemented with 2% fetal calf serum (FCS) (1 ml well–1 for 24-well cell culture plates). Incubate at 20°C.

Monitoring incubation: Follow the course of infection in positive controls and other inoculated cell cultures by microscopic examination at ×40–100 magnification for 7 days. The use of a phase-contrast microscope is recommended.

Maintain the pH of the cell culture medium at between 7.3. and 7.6. during incubation. This can be achieved by the addition to the inoculated medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) or 2 M Tris (Tris [hydroxymethy]) aminomethane)/HCl buffer solution (for cell culture plates).

The cytopathic effect (CPE) is characterised by rounding, detachment and lysis of cells (Fijan, 1999). If a CPE appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures must be undertaken immediately.

Subcultivation procedures: Using a pipette, try to dislodge cells from the cell culture vessels and collect aliquots of cell culture medium plus cells from all inoculated monolayers, keeping different groups separate. The aliquots of the 1/100 and 1/000 dilutions are pooled and inoculated on to fresh 24 hour-old cell cultures to effect 1/10 and 1/100 final dilutions of the pooled aliquots. Incubate and monitor as described above.

If no CPE occurs the test may be declared negative. However, if undertaking surveillance to demonstrate freedom from SVCV it would be advisable to screen the cells at the end of the 14 days using an SVCV-specific RT-PCR (Section 4.4). Following a positive result culture should be re-attempted.

Following isolation, the virus must be identified, and this can be achieved by antigen detection methods, virus neutralisation or nucleic acid identification methods. The former two methods are generally regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other viruses occur. Commercially available kits using polyclonal antibodies may also lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005). Nucleic acid detection methods must always be followed up by sequencing or use of a method such as reverse hybridisation (Sheppard *et al*., 2007) to confirm the identity of the virus.

4.4. Nucleic acid amplification

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.

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.

4.4.3. Other nucleic acid amplification methods

Loop-mediated isothermal amplification assays are available to detect and confirm SVCV infections (Shivappa *et al*., 2008), however, they are currently not recommended as they are not sufficiently validated.

SVCV has also been detected using RT-PCR and hybridisation with non-radioactive probes to determine the genotype (Sheppard *et al*., 2007), however, it is currently not recommended as it is not sufficiently validated.

4.5. Amplicon sequencing

Nucleotide sequencing of all RT-PCR amplicons (Section 4.4.2) is recommended as one of the final steps for confirmatory diagnosis. SVCV-specific sequences will share a higher degree of nucleotide similarity to one of the published reference sequences for SVCV (Genbank accession U18101, AJ318079, DQ097384 and EU177782) compared to the published reference sequences for the Pike spriviviruses (GenBank FJ872827, KC113518 and KC113517).

4.6. *In-situ* hybridisation

Although *in-situ* hybridisation can be used to locate SVCV in different tissues in known positive animals, this assay is currently not recommended as it has not been validated as a diagnostic tool for the detection of SVCV.

4.7. Immunohistochemistry

SVCV can be detected by immunohistochemistry, however, care must be taken with interpreting the results of these tests for SVCV, and positive results should be confirmed by RT-PCR and sequencing.

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Store and transport the kidney pieces as indicated in Section 2.2.1 of Chapter 2.3.0 *General information* (diseases of fish) together with the other organs required for virus isolation.

iv) Allow the imprint to air-dry for 20 minutes.

v) Fix with cold acetone (stored at –20°C) for glass slides or 80% acetone in water or 30% acetone in ethanol, also at –20°C, for plastic wells. Let the fixative act for 15 minutes. Allow the imprints to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

vi) Rehydrate the imprints if they have been stored frozen by four rinsing steps with PBS containing 0.05% Tween 20 (PBST), and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

vii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.

viii) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).

ix) Incubate the imprints with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.

x) Rinse four times with PBST.

xi) Incubate the imprints with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xii) Rinse four times with PBST.

xiii) View the treated imprints on plastic plates immediately, or mount the slides with cover-slips using glycerol saline at pH 8.5, or a commercially-available mountant.

xiv) Examine under a fluorescence microscope with ×10 eye pieces and ×20 or ×40 objective lenses having numerical aperture of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

4.8. Bioassay

Not available.

4.9. Antibody- or antigen-based detection methods

Antibody- or antigen-based methods that detect SVCV must be regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other closely related spriviviruses (PFRV, GrCRV and TenRV) may occur. Commercially available kits using polyclonal antibodies may lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005). These techniques should not be used as a screening method.

4.9.1. Antigen enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, pH 9.5 (200 µl well^{-1}). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse four times with PBST.

iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl well^{-1}).

v) Rinse four times with PBST.

vi) Add 2% non-ionic detergent (Triton X-100 or Nonidet P-40) to the virus suspension to be identified.

vii) Dispense 100 µl well^{-1} of two- or four-step dilutions of the virus to be identified, and of the non-infected cell culture harvest (negative control). Also include SVCV positive control virus. Incubate for 1 hour at 37°C.

viii) Rinse four times with PBST.

ix) Add to the wells, 200 µl of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG to SVCV. An MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin can also be used. Incubate for 1 hour at 37°C.

x) Rinse four times with PBST.

xi) If HRPO-conjugated antibody has been used, go to step xiii. Otherwise, add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C.

xii) Rinse four times with PBST.

xiii) Add 200 µl of a suitable substrate and chromogen, such as tetramethylbenzidine dihydrochloride. Stop the course of the test when positive controls react, and read the results.

Enzyme-linked immunosorbent assay (ELISA) *using tissue homogenates*

See Section 2.2.2 of Chapter 2.3.0 *General information* (diseases of fish) for obtaining organ homogenates.

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, pH 9.5 (200 µl well^{-1}). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively.

For the identification of SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse four times with PBST.

iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl well^{-1}).

v) Rinse four times with PBST.

vi) Store a 1/4 aliquot of each homogenate at 4°C, in case the test is negative and virus isolation in cell culture is required.

vii) Treat the remaining part of the homogenate with 2% Triton X-100 or Nonidet P-40 and 2 mM of phenyl methyl sulphonide fluoride; mix gently.

viii) Dispense 100 µl well^{-1} of two- or four-step dilutions of the sample to be identified, and of negative control tissues. Also include an SVCV positive control virus. Incubate for 1 hour at 37°C.

ix) Rinse four times with PBST.

x) Add to the wells, 200 µl of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG to SVCV. A MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin can also be used. Incubate for 1 hour at 37°C.

xi) Rinse four times with PBST.

xii) If HRPO-conjugated antibody has been used, go to step xiv. Otherwise, add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C.

xiii) Rinse four times with PBST.

xiv) Add 200 µl of a suitable substrate and chromogen, such as tetramethylbenzidine dihydrochloride. Stop the course of the test when positive controls react, and read the results.

xv) If the test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as described in Section 4.3.

4.9.2. Indirect fluorescent antibody test (IFAT)

i) Prepare monolayers of cells in 2 cm^2 wells of plastic cell culture plates, flasks or on cover-slips or glass slides in order to reach approximately 80% confluency within 24 hours of incubation at 25°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FCS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks. For tests using cells cultured on glass cover-slips or slides, the dilutions are made in sterile containers and then used to inoculate the cells.

iii) Dilute the control virus suspension of SVCV in a similar way, in order to obtain a virus titre of about 5000–10,000 PFU ml^{-1} in the cell culture medium.

iv) Incubate at 20°C for 24 hours.

v) Remove the cell culture medium, rinse once with 0.01 M phosphate-buffered saline (PBS), pH 7.2, then three times briefly with cold acetone (stored at –20°C) for slides or cover-slips or 80% acetone in water or 30% acetone in ethanol, also at –20°C, for cells on plastic substrates. Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 cm^2 of cell monolayer.

vi) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

vii) Rehydrate the dried cell monolayers, if they have been stored frozen, by four rinsing steps with PBST and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

viii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.

ix) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).

x) Incubate the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.

xi) Rinse four times with PBST.

xii) Incubate the cell monolayers with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) View the treated cell monolayers on plastic substrates immediately, or mount the slides or cover-slips using glycerol saline at pH 8.5, or a commercially available mountant.

xv) Examine under a fluorescence microscope with ×10 eye pieces and ×20 or ×40 objective lenses having numerical apertures of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

4.10. Other methods

Not applicable.

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.

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 of clinical signs (Section 6.2) 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.

1 For example transboundary commodities.

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) SVCV-typical CPE. in cell culture

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with SVCV is considered to be confirmed if the following criterion is met:

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

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 antigen ELISA

iv) Positive result by IFAT

v) Positive result by immunohistochemistry

vi) 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 the following criterion is met:

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

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

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

6.3.2. For surveillance of apparently healthy animals

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.

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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 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.