CHAPTER 2.3.10.

INFECTION WITH VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS

1. Scope

Infection with viral haemorrhagic septicaemia virus (VHSV) means infection with the pathogenic agent viral haemorrhagic septicaemia virus of the Genus *Novirhabdovirus* and Family *Rhabdoviridae.*

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

VHSV is a bullet-shaped particle, approximately 70 nm in diameter and 180 nm in length, that contains a negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides, and possesses an envelope that contains the membrane glycoprotein, which is the neutralising surface antigen. The genome encodes six proteins: a nucleoprotein N; a phosphoprotein P (formerly designated M1); a matrix protein M (formerly designated M2); a glycoprotein G; a non-virion protein NV and a polymerase L (Walker *et al*., 2000).

G-gene nucleotide sequences have been used to classify VHSV isolates into four major genotypes (I, II, III and IV) and nine subtypes (Ia–Ie and IVa-IVd) with almost distinct geographical distributions (Einer-Jensen *et al*., 2004; Elsayed *et al*., 2006). The host range and the pathogenicity appear, at least to some extent, to be linked to the genotype of VHSV.

i) Genotype Ia

Almost all VHSV isolates causing outbreaks in European rainbow trout (*Oncorhynchus mykiss*) farms cluster in sub-lineage Ia, of which isolates have been reported from most continental European countries (Einer-Jensen *et al*., 2004; Kahns *et al*., 2012; Snow *et al*., 2004; Toplak *et al*., 2010). However, genotype Ia isolates have also been detected in other finfish species in Europe such as brown trout (*Salmo trutta*), pike (*Esox lucius*) and grayling (*Thymallus thymallus*) (de Kinkelin & Le Berre, 1977; Jonstrup *et al*., 2009). Genotype Ia isolates have generally caused outbreaks in freshwater-farmed rainbow trout, but isolates have also been obtained from sea-reared rainbow trout and turbot (*Scophthalmus maximus*) (Schlotfeldt *et al*., 1991; Snow *et al*., 2004). Genotype Ia can be further subdivided into two major subpopulations, Ia-1 and Ia-2, with a distinct geographic distribution within Europe (Kahns *et al*., 2012).

ii) Genotype Ib

The isolates included in this genotype have been obtained from finfish in the marine environment in the Baltic Sea, Kattegat, Skagerrak, the North Sea and the English Channel (Einer-Jensen *et al*., 2004; Skall *et al*., 2005b; Snow *et al*., 2004) and as far north as latitude 70°N close to Nordkapp in Norway (Sandlund *et al*., 2014). A single case was observed in Japan (Nishizawa *et al*., 2002). None of the isolations from wild fish has been associated with clinical disease outbreaks (Johansen *et al*., 2013). Genotype Ib has been associated with evidence of transfer between wild fish and farmed rainbow trout in only two cases in pen-reared rainbow trout in Sweden in 1998 and 2000 (Nordblom, 1998; Nordblom & Norell, 2000; Skall *et al*., 2005a).

iii) Genotype Ic

This genotype consists of a smaller group of Danish isolates from freshwater farmed rainbow trout. Isolates of this genotype have also been detected in Germany and Austria (Jonstrup *et al*., 2009).

iv) Genotype Id

The isolates included in this genotype consist of some old Scandinavian isolates from the 1960s and from outbreaks of infection with VHSV in Finland in sea-reared rainbow trout in 2000. These outbreaks occurred in two different areas and all of the isolates sampled were clustered in the Id

genotype group. In infection trials, it was demonstrated that the isolates were pathogenic to rainbow trout, but less virulent than most Ia isolates (Raja-Halli *et al*., 2006).

v) Genotype Ie

The isolates included in this genotype have been obtained from both freshwater and marine (the Black Sea) environments in Georgia and Turkey. Isolations were from both farmed and wild turbot (Jonstrup *et al*., 2009; Kalayci *et al*., 2006; Nishizawa *et al*., 2006) and from rainbow trout (Einer-Jensen *et al*., 2004). VHSV Ie has also been isolated from whiting (*Merlangius merlangus*) and sea bass (*Dicentrarchus labrax*) in the Black Sea (Altuntas & Ogut, 2010).

vi) Genotype II

The isolates included in this genotype have been primarily detected in marine wild finfish, in particular Atlantic herring (*Clupea harengus*), from the Baltic Sea, including the Gulf of Bothnia and the Gulf of Finland, (Gadd *et al*., 2011; Snow *et al*., 2004). Genotype II isolates have also been detected in lamprey (*Lampetra fluviatilis*) caught in freshwater from the rivers Kalajoki and Lestijoki, which have an outlet into the Gulf of Bothnia (Gadd *et al*., 2010).

vii) Genotype III

The isolates included in this genotype originate from wild and farmed finfish in the North Atlantic Sea from the Flemish Cap (Lopez-Vazquez *et al*., 2006b) to the Norwegian coast (Dale *et al*., 2009), the North Sea around the British Isles, Skagerrak and Kattegat. Outbreaks of infection with VHSV in sea-farmed turbot in the United Kingdom and Ireland in the 1990s were attributed to infection with genotype III isolates, and in 2007 an outbreak in sea-reared rainbow trout at the Norwegian west coast was due to VHSV genotype III. Outbreaks of infection with VHSV in five species of wrasse used as cleaner fish around the Shetland Islands were also due to this genotype (Munro *et al*., 2015).

viii) Genotype IVa

The isolates included in this genotype have been detected in finfish from the coastal environments of North America spanning from California to Alaska in the west and around the northeastern United States up through Newfoundland, Canada. This genotype has also been reported from the Asian countries of South Korea and Japan. Genotype IVa isolates in North America have caused severe epidemics in numerous wild marine species such as Pacific herring (*Clupea pallasii pallasii*) (Meyers & Winton, 1995), which can serve as a reservoir of virus to sympatric sea-reared Atlantic salmon (*Salmo salar*) (Garver *et al*., 2013). In Asia, genotype IVa isolates have caused disease outbreaks in bastard halibut (*Paralichthys olivaceus*) (Ogut & Altuntas, 2014).

ix) Genotype IVb

The isolates included in this genotype have been detected in finfish in fresh water from the North America Laurentian Great Lakes region (Gagne *et al*., 2007; Thompson *et al*., 2011; Winton *et al*., 2008) and have caused die-offs in numerous fish species (Faisal & Winters, 2011).

x) Genotype IVc

The isolates included in this genotype have been detected from finfish from the estuarine waters of New Brunswick and Nova Scotia, Canada (Gagne *et al*., 2007; Pierce & Stepien, 2012; Stepien *et al*., 2015).

xi) Genotype IVd

The isolates included in this genotype have been detected in Iceland where they were identified in wild and sea-farmed lumpfish (*Cyclopterus lumpus*) (Gudmundsdottir *et al*., 2019).

2.1.2. Survival and stability in processed or stored samples

VHSV survival in host tissue is dependent on the conditions for storage. VHSV can remain infectious for long time periods while stored frozen in fish tissue. However, VHSV-infected fish subjected to the commercial freezing process (core block temperature of –24°C) had a 90% reduction in viral titre after the tissue was thawed (Arkush *et al*., 2006). VHSV is sensitive to enzymatic degradation, environments with high bacterial load and high temperatures (above 28°C). Fresh (unfrozen) muscle tissue from VHSV-infected rainbow trout could transmit VHSV to naïve fish (Oidtmann *et al.,* 2011a). VHSV is tolerant of high salt concentrations such as in brine-treated fish (Skall *et al.,* 2015) or while stored in concentrated ammonium sulphate solution (Pham *et al*., 2018). For optimal retention of VHSV in fish tissue, the sample

should be placed in transport medium with antibiotics and kept on ice without freezing and processed within 24 hours after sampling.

2.1.3. Survival and stability outside the host

VHSV survival outside the host is dependent on the physico-chemical conditions of the aqueous medium (Ahne, 1982) and on temperature: the virus survives for longer periods at 4°C compared with 20°C (Parry & Dixon, 1997).

VHSV is significantly more stable in freshwater than seawater. The virus has been documented to persist in freshwater for 28–35 days at 4°C (Parry & Dixon, 1997) and has been found to be infective for 1 year at 4°C in filtered freshwater (Hawley & Garver, 2008). In raw freshwater at 15°C, the 99.9% inactivation time was 13 days, but in seawater the virus was inactivated within 4 days (Hawley & Garver, 2008). In another study using seawater at 15°C, the infectivity of the virus was reduced by 50% after 10 hours, but could still be recovered after 40 hours (Kocan *et al.,* 2001). There appears to be no consistent correlation between the origin and stability of the virus isolates: freshwater isolates are not always the most stable in freshwater and seawater isolates are not consistently more stable in seawater (Hawley & Garver, 2008).

The virus remains stable for a longer time if sterile organic materials are added to the water, such as ovarian fluids or blood products, such as bovine serum (Kocan *et al.,* 2001). When the seawater was sterilised by autoclaving, or when passed through a 0.22 µm membrane, virus survival was prolonged significantly (60 days at 15°C and 32 days at 20°C), suggesting the bacterial load in the water is an important factor of viral decay.

2.2. Host factors

2.2.1. Susceptible host species

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

Family	Scientific name	Common name	Genotype
Ammodytidae	*Ammodytes hexapterus*	Pacific sand lance	IVa
Aralichthyidae	*Paralichthys olivaceus*	Bastard halibut	IVa
Carangidae	*Trachurus mediterraneus*	Mediterranean horse mackerel	Ie
Centrarchidae	*Ambloplites rupestris*	Rock bass	IVb
	Lepomis gibbosus	Pumpkinseed	IVb
	Lepomis macrochirus	Bluegill	IV, IVb
	Micropterus dolomieu	Smallmouth bass	IVb
	Micropterus salmoides	Largemouth bass	IVb
	Pomoxis nigromaculatus	Black crappie	IVb
Clupeidae	*Alosa immaculata*	Pontic shad	Ie
	Sardina pilchardus	Pilchard	ND
	Clupea harengus	Atlantic herring	Ib, III
	Clupea pallasii pallasii	Pacific herring	IVa
	Dorosoma cepedianum	American gizzard shad	IVb
	Sardinops sagax	South American pilchard	IVa
	Sprattus sprattus	European sprat	Ib
Cyclopteridae	*Cyclopterus lumpus*	Lumpfish	IVd
Cyprinidae	*Danio rerio*	Zebra fish	IVa
	Notropis hudsonius	Spottail shiner	IVb
	Notropis atherinoides	Emerald shiner	IVb
	Pimephales notatus	Bluntnose minnow	IVb
	Pimephales promelas	Fathead minnow	IVb
Embiotocidae	*Cymatogaster aggregata*	Shiner perch	IVa

Family	Scientific name	Common name	Genotype
Engraulidae	*Engraulis encrasicolus*	European anchovy	Ie
Esocidae	*Esox lucius*	Northern pike	Ia, IVb
	Esox masquinongy	Muskellunge	IVb
Fundulidae	*Fundulus heteroclitus*	Mummichog	IVc
Gadidae	*Gadus macrocephalus*	Pacific cod	IVa
	Gadus morhua	Atlantic cod	Ib, III
	Merlangius merlangus	Whiting	Ie
	Micromesistius poutassou	Blue whiting	Ib, III
	Trisopterus esmarkii	Norway pout	Ib, III
Gasterosteidae	*Gasterosteus aculeatus*	Three-spine stickleback	IVc
Gobiidae	*Neogobius melanostomus*	Round goby	IVb
	Pomatoschistus minutus	Sand goby	Ib
Ictaluridae	*Ameiurus nebulosus*	Brown bullhead	IVb
Labridae	*Centrolabrus exoletus*	Rock cook wrasse	III
	Ctenolabrus rupestris	Goldsinny wrasse	III
	Labrus bergylta	Ballan wrasse	III
	Labrus mixtus	Cuckoo wrasse	III
	Symphodus melops	Corkwing wrasse	III
Lotidae	*Gaidropsarus vulgaris*	Three-bearded rockling	Ie
Moronidae	*Morone americana*	White perch	IVb
	Morone chrysops	White bass	IVb
	Morone saxatilis	Striped bass	IVb, IVc
Mullidae	*Mullus barbatus*	Red mullet	Ie
Osmeridae	*Thaleichthys pacificus*	Eulachon	IVa
Percidae	*Sander vitreus*	Walleye	IVb
	Perca flavescens	Yellow perch	IVb
Petromyzontidae	*Lampetra fluviatilis*	River lamprey	II
Pleuronectidae	*Limanda limanda*	Common dab	Ib
	Platichthys flesus	European flounder	Ib
	Pleuronectes platessus	European plaice	III
Rajidae	*Raja clavata*	Thornback ray	Ie
Salmonidae	*Coregonus artedii*	Lake cisco	IVb
	Coregonus clupeaformis	Lake whitefish	IVb
	Coregonus lavaretus	Common whitefish	Ia
	Oncorhynchus kisutch	Coho salmon	IVa
	Oncorhynchus mykiss	Rainbow trout	Ia-e, III, IVb
	Oncorhynchus mykiss X Oncorhynchus kisutch hybrids	Rainbow trout X coho salmon hybrids	Ia
	Oncorhynchus tshawytscha	Chinook salmon	IVa, IVb
	Salmo marmoratus	Marble trout	Ia
	Salmo salar	Atlantic salmon	Ia, Ib, II, III, IVa
	Salmo trutta	Brown trout	Ia, Ib
	Salvelinus namaycush	Lake trout	Ia, IVa, IVb
	Thymallus thymallus	Grayling	Ia
Scophthalmidae	*Scophthalmus maxima*	Turbot	Ib, III
Sciaenidae	*Aplodinotus grunniens*	Freshwater drum	IVb

Family	Scientific name	Common name	Genotype
Scombridae	*Scomber japonicus*	Pacific chub mackerel	IVa
Soleidae	*Solea senegalensis*	Senegalese sole	III
Uranoscopidae	*Uranoscopus scaber*	Atlantic stargazer	Ie

ND: Not determined.

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with VHSV according to Chapter 1.5 of the *Aquatic Code* are:

Family	Scientific name	Common name	Genotype
Adrianichthydiae	*Oryzias latipes*	Japanese rice fish	IVb
	Oryzias dancena	Marine medaka	IVa
Ammodytidae	*Ammodytes personatus*	Sandeel	Ib
Anguillidae	*Anguilla anguilla*	European eel	III
Argentinidae	*Argentina sphyraena*	Lesser Argentine	Ib
Belonidae	*Belone belone*	Garfish	Ie
Carangidae	*Seriola dumerili*	Greater amberjack	IVa
Catostomidae	*Catostomus commersonii*	White sucker	IVb
	Moxostoma anisurum	Silver redhorse	IVb
	Moxostoma macrolepidotum	Shorthead redhorse	IVb
Centrarchidae	*Pomoxi annuluris*	White crappie	IVb
Clupeidae	*Alosa pseudoharengus*	Alewife	IVb
Cottidae	*Cottus pollux*	Japanese fluvial sculpin	IVb
Cyprinidae	*Semotilus corporalis*	Fallfish	IVb
	Notemigonus crysoleucas	Golden shiner	IVb
Esocidae	*Esox lucius* X *E. masquinongy* hybrids	Tiger muskellunge (*Esox masquinongy* X *E. lucius* or *E. lucius* X *E. masquinongy*)	IVb
Fundulidae	*Fundulus diaphanus*	Banded killifish	IVb
Gadidae	*Gadiculus argenteus*	Silvery pout	Ib
	Melanogrammus aeglefinus	Haddock	III
	Theragra chalcogramma	Alaska pollock	IVa
	Trisopterus minutus	Poor cod	III
Ictaluridae	*Ictalurus punctatus*	Channel catfish	IVb
Liparidae	*Liparis tessellatus*	Cubed snailfish	IV
Lotidae	*Lota lota*	Burbot	IVb
	Enchelyopus cimbrius	Fourbeard rockling	Ib
Merlucciidae	*Merluccius productus*	North Pacific hake	IVa
Moronidae	*Dicentrarchus labrax*	European sea bass	Ia
Mugilidae	*Mugil cephalus*	Flathead grey mullet	IV
Ophidiidae	*Hoplobrotula armata*	Armoured cusk	IV
Osmeridae	*Hypomesus pretiosus*	Surf smelt	ND
Oxudercidae	*Rhinogobius* sp. (undescribed species)	Yoshinobori	IVb
Percopsidae	*Percopsis omiscomaycus*	Trout perch	IVb
Petromyzontinae	*Petromyzon marinus*	Sea lamprey	IVb
Pleuronectidae	*Glyptocephalus stelleri*	Blackfin flounder	IVa

Family	Scientific name	Common name	Genotype
	Hippoglossus hippoglossus	Atlantic halibut	III
	Reinhardtius hippoglossoides	Greenland halibut	III
Salmonidae	*Oncorhynchus mykiss* X *Salvelinus alpinus* hybrids	Rainbow trout X Arctic charr hybrids	Ia
	Oncorhynchus mykiss X *Salvelinus namaycush* hybrids	Rainbow trout X lake trout hybrids	Ia
	Oncorhynchus mykiss X *Salmo trutta hybrids*	Rainbow trout X brown trout hybrids	Ia
	Salvelinus alpinus	Arctic charr	Ia
	Salvelinus fontinalis	Brook trout	Ie
Sciaenidae	*Larimichthys polyactis*	Yellow croaker	IV
Scorpaenidae	*Scorpaena porcus*	Black scorpionfish	Ie
	Scorpaena izensis	Izu scorpionfish	IV
Scyliorhinidae	*Scyliorhinus torazame*	Claudy catshark	IV
Stromateidae	*Pampus argenteus*	Silver pomfret	IV
Trichiuridae	*Trichiums lepturus*	Largehead hairtail	IV
Triglidae	*Eutrigla gurnardus*	Gray gurnard	III

ND: Not determined.

In addition, pathogen-specific positive reverse-transcription polymerase chain reaction (RT-PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: Sablefish (*Anoplopoma fimbria*).

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

Rainbow trout is the most susceptible species to VHSV infection with genotype Ia. For VHSV genotypes Ib, II and III, shoaling wild-living species such as Atlantic herring and European sprat are likely to be the natural hosts, while for genotype IVa, Pacific herring is the natural host. VHSV genotype III has caused disease in farmed turbot and wrasse and genotype IVa in sea-farmed Atlantic salmon, turbot, and bastard halibut.

Infection with VHSV may cause disease and mortality in all life stages of susceptible fish. VHSV does not infect fish eggs (Munro & Gregory, 2010).

In surveys of wild marine fish, VHSV has been isolated from most year classes. Few fry have been tested however, as they are usually not caught during the surveys. The highest prevalence of virus in sampled wild populations was found in shoaling fish, such as Atlantic herring, European sprat and Norway pout (Skall *et al*., 2005a).

For the purposes of Table 4.1 rainbow trout alevin and fry (e.g. up to approximately 1 g in weight) may be considered early life stages, fingerlings and ongrowing fish up to 50 g be considered as juveniles and fish over 50 g adults.

2.2.4. Distribution of the pathogen in the host

In fish showing clinical signs, the virus is abundant in all tissues including gill, skin and muscles (Sandlund *et al.,* 2014). Target organs are anterior kidney, heart and spleen, as these are the sites in which virus is most abundant. In chronic stages, virus titres can become high in the brain (Smail & Snow, 2011; Wolf, 1988).

2.2.5. Aquatic animal reservoirs of infection

Some survivors of epizootics will become long-term carriers of the virus. Pacific herring surviving infection with VHSV genotype IVa have transmitted disease to naïve cohabitants (Gross *et al*., 2019). Almost all isolations of VHSV genotype Ib, II and III from wild fish species are from individuals with no clinical signs of infection with VHSV and with low virus titres (Skall *et al.,* 2005a).

2.2.6. Vectors

VHSV has been detected in numerous species of animals, which are not susceptible species and may therefore act as vectors. However, transmission of VHSV by vectors has not been demonstrated. VHSV has been isolated from common snapping turtle (*Chelra serpentina*), leech (*Myzobdella lugubris*), northern map turtle (*Grapetemys geographicas*) and water flea (*Moina macrocopa*) and these species may be potential vectors for transmission of VHSV (Faisal & Schultz, 2009; Goodwin & Merry, 2011; Ito & Olesen, 2017). VHSV has also been isolated from the amphipods *Hyalellea* spp. and *Diporeia* spp., suggesting that benthic macroinvertebrates may be vectors for VHSV IVb in endemically affected systems. In contrast VHSV was not detected in mussels or sediments in the same water environment (Faisal & Winters 2011; Throckmorton *et al*., 2017). VHSV has also been isolated from leech, *Myzobdella lugubris,* in the Great Lakes (Faisal & Schulz, 2009; Faisal & Winters, 2011).

Piscivorous birds may act as VHSV vectors by carrying the virus, for example, on their beaks and feet (Olesen & Jorgensen, 1982), or through regurgitation of infected fish (Peters & Neukirch, 1986).

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Mortality varies, depending on many environmental and physiological conditions, most of which have not been fully determined. The disease is, in general, a cool or cold water disease with highest mortality at temperatures around 9–12°C. Small rainbow trout fry (0.3–3 g) are most susceptible to genotype Ia with mortalities close to 100%, but all sizes of rainbow trout can be affected with mortalities ranging from 5 to 90% (Skall *et al.*, 2004). Immersion infection trials also induced up to 100% mortality in Pacific herring when challenged with genotype IVa (Hershberger *et al.,* 2010a). Mortality in wild finfish also varies from no observable deaths to severe die-offs. The prevalence of VHSV genotype Ib, II and III varies from 0 to 16.7% in Northern European waters (Skall *et al.*, 2005b).

2.3.2. Clinical signs, including behavioural changes

The occurrence of the following clinical signs is characteristic of infection with VHSV: rapid onset of mortality, lethargy, darkening of the skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of the fins or in the gills, eyes or skin, abnormal swimming such as flashing and spiralling, and a distended abdomen due to oedema in the peritoneal cavity. In rainbow trout, the clinical appearance is typically lethargic dark fish with exophthalmia at the pond shores and the outlet. Characteristically, diseased fish will not attempt to escape when netted.

Infection with some genotypes of VHSV results in predominant clinical signs in some susceptible species. Skin lesions in cod and herring from the Pacific and Atlantic Oceans (including the North Sea), and in haddock from the North Sea, have been described frequently (Jensen & Larsen, 1979; Meyers *et al.,* 1992; Meyers & Winton, 1995; Smail, 2000; Vestergard Jorgensen & Olesen, 1987). In farmed bastard halibut, an 'anaemic' form (pale gills) of infection with VHSV has also been described (Isshiki *et al.,* 2001).

2.3.3 Gross pathology

Gross pathology includes generalised petechial haemorrhaging in the skin, muscle tissue (especially in dorsal muscles) and internal organs. It is important to examine the dorsal musculature for the presence of petechial bleeding, which is a very common sign of infection with VHSV. The kidney is dark red in the acute phase and can demonstrate severe necrosis in moribund fish. The spleen is moderately swollen. The liver is often pale and mottled. The gastrointestinal tract, especially the hindgut, is pale and devoid of food.

2.3.4. Modes of transmission and life cycle

Transmission primarily occurs horizontally through water, with excretion of virus in the urine, and directly from the skin (Smail & Snow, 2011). Oral transmission was also demonstrated indicating that preying on infected fish and vectors may transfer the disease (Schonherz *et al.* 2012).

Experimentally it has been demonstrated that feeding fresh (unfrozen) muscle tissue from VHSV-infected rainbow trout can transmit VHSV to naïve fish (Oidtmann *et al.,* 2011a).

There are no indications or evidence of true vertical transmission of VHSV (Bovo *et al*., 2005a; Munro & Gregory, 2010).

2.3.5. Environmental factors

Disease generally occurs at temperatures between 4°C and 14°C. At water temperatures between 15°C and 18°C, the disease generally takes a short course with low levels of mortality.

Low water temperatures (1–5°C) generally result in an extended disease course with low daily mortality but high accumulated mortality. Outbreaks of infection with VHSV occur during all seasons but are most common in spring when water temperatures are rising or fluctuating.

Field observations and experimental studies suggest that warmer water temperatures greatly reduce or inhibit transmission. Natural outbreaks of infection with VHSV are not observed at water temperatures greater than 18°C. In challenge trials, fish exposed to VHSV and reared at temperatures below 15°C displayed high mortality whereas those infected and reared at 20°C did not (Arkush *et al.,* 2006; Castric & de Kinkelin, 1984). For more detailed reviews, see Wolf (1988) and Smail & Snow (2011).

2.3.6. Geographical distribution

Infection with VHSV has been reported from countries in Europe, North Amercia and North Asia. Some countries in these regions have declared freedom from infection with VHSV. The disease has never been reported from the Southern Hemisphere.

For recent information on distribution at the country level consult the WAHIS interface (https://wahis.oie.int/#/home).

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Although research on vaccine development for VHSV has been ongoing for more than four decades, a commercial vaccine is not yet available. Candidate vaccines have included killed vaccines, attenuated live vaccines, a recombinant vaccine in prokaryotic and eukaryotic expression systems, and DNA-based vaccines. For a review see Lorenzen & LaPatra (2005). No vaccines currently affect the diagnostic sensitivity and specificity of tests for infection with VHSV.

2.4.2. Chemotherapy including blocking agents

No therapies are currently available.

2.4.3. Immunostimulation

Several immunostimulants, such as yeast-derived beta-glucans, IL-1β-derived peptides, and probiotics have been assessed for enhancing protection against infection with VHSV (Peddie *et al*., 2003). Several researchers report positive effects, but no immunostimulant directed specifically at enhanced resistance to infection with VHSV is available. Furthermore, it remains unknown as to whether their use can affect sensitivity and specificity of VHSV assays.

2.4.4. Breeding resistant strains

Additive genetic variation in rainbow trout for resistance to infection with VHSV has been demonstrated (Dorson *et al*., 1995; Henryon *et al*., 2002a; 2002b). In a study by Henryon *et al*. (2005), the heritability of resistance to VHSV was 0.11 for time to death on a logarithmic timescale. Identification of a major quantitative trait loci (QTL) for VHSV resistance in rainbow trout may pave the way for genetic selection for VHSV resistant fish (Verrier *et al.,* 2013), however, no resistant rainbow trout strains are commercially available.

2.4.5. Inactivation methods

VHSV is sensitive to a number of common disinfectants (e.g. UV light, chlorine, iodophore, sodium hypochlorite), to temperatures above 30°C, to bacterial degradation in sediments and enzymatic activity in decomposing fish. For a review see Bovo *et al*., 2005b.

2.4.6. Disinfection of eggs and larvae

Disinfection of newly fertilised or eyed eggs is an efficient and cost-effective preventive measure for stopping the spread of the disease in salmonids (for the recommended protocol see Chapter 4.4. of the *Aquatic Code*).

2.4.7. General husbandry

Poor water quality, high fish density, high feeding rate, infection with other diseases such as proliferative kidney disease, ichthyophthiriasis, bacterial kidney disease, etc. can influence the course and severity of infection with VHSV. In general, an increase in temperature, restricted feeding, reduced fish density and restricted handling may reduce mortality. In endemically infected farms, stocking with naïve fry is usually done when the water temperature is at near maximum levels.

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

Clinical inspections should be carried out during a period when the water temperature is below 14°C or whenever the water temperature is likely to reach its lowest annual point. 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.

Fish to be sampled are selected as follows:

i) For genotype I, in farms where rainbow trout are present, fish of that species should be selected for sampling. If rainbow trout are not present, the sample should be obtained from fish of all other VHSV-susceptible species present (as listed in Table 2.1) or from species with incomplete evidence for susceptibility (as listed in Table 2.2). However, the species should be proportionally represented in the sample. For other genotypes (II, III, and IV), species of known susceptibility to the genotype in question should be sampled.

ii) Susceptible species should be sampled following risk-based criteria for targeted selection of populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or introduction of stocks of unknown risk status).

iii) If more than one water source is used for fish production, fish from all water sources should be included in the sample.

3.2. Selection of organs or tissues

In populations with clinical disease, the optimal tissues are anterior kidney, spleen and heart (Lovy *et al.,* 2012; Oidtmann *et al.,* 2011).In apparently healthy populations, the optimal tissues are anterior kidney and heart and, during the chronic phase of infection, brain, as VHSV can persist in tissues of the nervous system (Hershberger, 2010b; Lovy *et al.,* 2012; Oidtmann *et al.,* 2011b).

When sampling fish too small in size to permit dissection of individual tissues, viscera including kidney should be collected or whole fish homogenised after removal of the body behind the anal pore. When sampling broodstock, ovarian fluid and milt can be taken.

3.3. Samples or tissues not suitable for pathogen detection

When possible, tissues with high enzymatic activity such as liver and viscera should be avoided as VHSV is very sensitive to enzymatic degradation. When performing cell culture assays tissues containing high bacteria counts, such as the intestine or skin, should be avoided to minimise risk of bacterial contamination of tissue culture cells. Preservatives and fixatives, such as RNAlater and formaldehyde can be toxic to tissue culture cells such as epithelioma papulosum cyprini (EPC) and fathead minnow (FHM), and can impact molecular detection methods (Auinger *et al.,* 2008; Pham *et al.,* 2018).

3.4. Non-lethal sampling

Fin and gill biopsies were shown to be effective nonlethal samples for detection of VHSV genotype IVb (Cornwell *et al.,* 2013) in clinically diseased fish and nested RT-PCR on blood samples from infected fish was also shown to be effective for VHSV detection (Lopez-Vazquez *et al.,* 2006a). In the case of brood fish, ovarian fluid and milt can be used for testing as an alternative to lethal testing. However, non-lethal sampling methods have not been fully validated for detection of all VHSV genotypes.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

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

Samples can be taken from the fish in accordance with the procedure described in Section 3.5.1, using a sterile instrument, and transferred to a sterile plastic tube containing transport medium.

Alternatively, samples may be placed in at least five volumes of RNA stabilisation reagents according to the recommendation from the manufacturers. Samples in RNA stabilising reagents can be shipped on ice or at room temperature if transport time does not exceed 24 hours.

Whole fish may also be sent to the laboratory (see Section 3.5.1).

Samples may also be frozen at –80°C and kept frozen until assayed (Siah *et al.,* 2014).

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

Tissue samples for histopathology should be fixed in 10% neutral buffered formalin immediately after collection. The recommended ratio of fixative to tissue is 10:1. To avoid excessive cross-linking, tissue should be transferred to ethanol after 24 hours if methods other than histopathology are used e.g. *in-situ* hybridisation.

3.5.4. Samples for other tests

If samples are processed for ELISA or other immunochemical assays, the procedures described in Section 3.5.1 for pathogen isolation should be followed.

3.6. Pooling of samples

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore, larger fish should be processed and tested individually. However, samples, especially fry or specimens up to 0.5 g, can be pooled to obtain enough material for virus isolation or molecular detection.

4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

+++ =	Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;
++ =	Suitable method(s) but may need further validation;
+ =	May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;
Shaded boxes =	Not appropriate for this purpose.

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities, and repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

Table 4.1. *OIE 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												
Immunohistochemistry[3]									++	++	++	2
Histopathology[3]						++	++	1				
Cell culture	+++	+++	+++	3	+++	+++	+++	3	+++	+++	+++	3
Real-time PCR	+++	+++	+++	3	+++	+++	+++	3	+++	+++	+++	3
Conventional RT-PCR	++	++	++	3	+++	++	+++	3	+++	++	+++	3
Amplicon sequencing[4]									+++	+++	+++	3
***In-situ* hybridisation**												
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA									+[5]	++[5]	++[5]	1
IFAT					++	++	++	2	++[5]	++[5]	++[5]	2
Serum neutralisation for Ab detection												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); RT-PCR = reverse-transcription polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; IFAT = indirect fluorescent antibody test;
[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.
[3]Histopathology and cytopathology can be validated if the results from different operators has been statistically compared.
[4]Sequencing of the PCR product.
[5]only for identification of cultured pathogen.
Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Not relevant.

4.2. Histopathology and cytopathology

The kidney, liver and spleen show extensive focal necrosis and degeneration – cytoplasmic vacuoles, pyknosis, karyolysis, and lymphocytic invasion. While the skeletal muscle does not appear to be a primary site of infection, erythrocytes can accumulate in the skeletal muscle bundles and fibres without causing damage to the muscle *per se* (Evensen *et al.,* 1994).

4.3. Cell culture for isolation

The recommended cell lines for VHSV detection are bluegill fry (BF-2), Chinook salmon embryo (CHSE-214), epithelioma papulosum cyprini (EPC) fathead minnow (FHM) or rainbow trout gonad (RTG-2). Susceptibility of a cell line to VHSV infection will depend on a range of parameters, including cell-line lineage or viral strain differences. Generally, VHSV isolates belonging to either genotypes I, II, or III culture best on BF-2 (Lorenzen *et al*., 1999), while genotype IV isolates culture best on the EPC cell line (US Department of the Interior, 2007).

4.3.1. Cell lines

Cell lines should be monitored regularly (e.g. every 6 months) to ensure that susceptibility to targeted pathogens has not changed.

Cells are grown at 20–24°C in a 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), or with HEPES-buffered medium (HEPES=N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid). The pH must be maintained at 7.6 ± 0.2. Cell cultures to be used for inoculation with tissue material should be young (4–48 hours old) and actively growing (not confluent) at inoculation. Cell susceptibility can be enhanced by reducing the amount of FBS to 2%. Pre-treatment of cells with 7% (w/v) PEG-20,000 solution (10–15 µl/cm^2) 15–30 minutes prior to sample inoculation has also been shown to increase detection of VHSV in culture (Batts *et al.,* 1991).

4.3.2. Sample preparation and inoculation

i) **Note:** Tissue and fluid samples should be kept cool throughout sample preparation procedures. Homogenise tissue samples using mortar and pestleor a tissue homogeniser. A small volume of medium (MEM-4 or HBSS [Hank's balanced salt solution] + antibiotics) may be needed to achieve complete homogenisation.

ii) Adjust the volume of medium to a final ratio of 10:1 (medium:tissue) and mix thoroughly. For fluid samples adjust the volume of medium to a final ratio of 1:1.

iii) Centrifuge the homogenate or fluid samples at 2000–4000 ***g*** for 15 minutes at 2–5°C.

iv) Remove the supernatant and pass through a 0.45 µM membrane filter (if available) or treat for either 4 hours at 15°C or overnight at 4°C with antibiotics, e.g. gentamicin 1 mg ml^{-1}.

If the sample cannot be inoculated within 48 hours after collection, the supernatant may be stored at –80°C provided virological examination is carried out within 14 days.

v) If samples originate from an area where infectious pancreatic necrosis virus (IPNV) is present, supernatants may be treated with IPNV antiserum. Mix the supernatant with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of IPNV and incubate for a minimum of one hour at 15°C or up to 18 hours at 4°C. The titre of the antiserum must be at least 1/2000 in a 50% plaque neutralisation test.

Treatment of all inocula with antiserum to IPNV aims at preventing cytopathic effect (CPE) caused by IPNV from developing in inoculated cell cultures. This will reduce the duration of the virological examination as well as the number of cases in which occurrence of CPE would have to be considered potentially indicative of VHSV. When samples come from production units that are considered free from infection with IPNV, treatment of inocula with antiserum to IPNV may be omitted.

vi) Samples are inoculated into cell cultures in at least two dilutions, i.e. the primary dilution and a 1:10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1:100 and 1:1000, respectively. The ratio between inoculum size and volume of cell culture medium should be about 1:10. For each dilution and each cell line, a minimum of about 2 cm^2 cell area, corresponding to one well in a 24-well cell culture tray, has to be used. Use of cell culture trays is recommended, but other units of similar or with larger growth area are also acceptable.

vii) Inoculated cell cultures are incubated at 15°C for 7–10 days. Using a microscope with 40–150× magnification, cultures should be inspected for toxicity the day after inoculation, particularly if supernatant was not filtered in step iv. The use of a phase-contrast microscope is recommended.

viii) Monitor the cells regularly (2–3 times a week) for the presence of CPE.

If CPE is observed, virus identification is required using tests recommended in Section 6. If no CPE is observed after the primary incubation period, subcultivation is performed.

Subcultivation

i) Remove cell culture supernatant from the primary culture and inoculate a newly (<48 hours) seeded cell culture plate.

ii) Incubate inoculated plates at 15°C and monitor for 7–10 days as described above.

If CPE is observed, virus identification is required using tests recommended in Section 6. If no CPE is observed after the primary incubation period or subcultivation, the sample is negative.

4.4. Nucleic acid amplification

Use of molecular tests (conventional RT-PCR and real-time RT-PCR) is common because of their rapidity, sensitivity and specificity. Real-time RT-PCR tests are generally more sensitive than conventional RT-PCR tests. The use of these tests for virus detection and identification during the acute stage of disease has been justified for a number of years. In the acute stage of infection, the sensitivity of some conventional RT-PCR (Kim *et al.,* 2018) and real-time RT-PCR tests (Garver *et al.,* 2011; Jonstrup *et al.,* 2013) is comparable to detection by cell culture and subsequent identification. The molecular methods described in this chapter are all targeting the nucleoprotein gene, as it is the highest transcribed gene in the VHSV genome (Chico *et al.,* 2006).

Recently, a novel one-step RT-PCR test was developed and validated (Kim *et al.,* 2018) to be used instead of the previously recommended conventional RT-PCR for detecting VHSV. This novel assay has a higher sensitivity detecting all VHSV genotypes, and outperforms the old method, particularly in detecting genotype IV.

For detecting all genotypes of VHSV with real-time RT-PCR, the methods of Jonstrup *et al*. (2013) and Garver *et al.* (2011) have been validated to stage 3, showing a sensitivity similar to detection by cell culture. These methods have high analytical and diagnostic sensitivity and specificity, and are robust across laboratories (Garver *et al.,* 2011; Jonstrup *et al.,* 2013; Warg *et al.,* 2014a; 2014b).

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

4.4.1. Real-time RT-PCR

Total RNA can be purified from: aliquots of cell culture medium from infected monolayer cells; or tissue/organs homogenised in MEM specified in Section 4.3.1, tissue samples in RNA stabilising reagent, fresh or frozen tissue samples, ovarian fluid.

In the case of culture medium from infected monolayer cells, or in tissue homogenised in MEM, aliquots should be centrifuged at 1000 ***g*** for 5 minutes to remove cell debris.

One-step (Jonstrup *et al.,* 2013) and two-step (Garver *et al.,* 2011) real-time RT-PCR assays targeting the nucleoprotein gene of VHSV have been stage 3 validated and are described herein.

Positive and negative controls should be included with each stage of the assay: extraction, reverse-transcription (two-step assay only) and real-time RT-PCR. An internal (endogenous) PCR control can be included however given the large number of fish species susceptible to infection with VHSV, the selection of an internal control is not trivial. If an endogenous control is to be used, primers and probes have to be designed, optimised and validated for each fish species to be tested.

Total RNA from infected cells and/or tissues is extracted using a phase-separation method (e.g. phenol-chloroform or Trizol) or by use of a commercially available RNA isolation kit used according to the manufacturer's instructions.

One-step real-time RT-PCR

In one-step RT-PCR gene-specific primers are used both to generate a cDNA transcript and for real-time RT-PCR. Both reactions occur in the same tube, which minimises the risk of contamination. The one-step real-time RT-PCR amplification can be performed using forward primer 5'-AAA-CTC-GCA-GGA-TGT-GTG-CGT-CC-3', reverse primer: 5'-TCT-GCG-ATC-TCA-GTC-AGG-ATG-AA-3', and FAM-labelled probe: 6'-FAM-TAG-AGG-GCC-TTG-GTG-ATC-TTC-TG-BHQ1. Primers are used at a final concentration of 900 nM and the final probe concentration is 250 nM. 5 µl of extracted RNA (50 ng–2 ug) is added to each 25 µl RT-PCR reaction. The assay was validated using Quantitect Probe RT-PCR kit (Qiagen, Germany) following the manufacturer's instructions and is recommended as other one-step kits have demonstrated reduced sensitivity (Jonstrup *et al.,* 2013). Thermal cycling conditions are 50°C for 30 minutes, 95°C for 15 minutes, 40 cycles of 94°C for 15 seconds, 60°C for 40 seconds, 72°C for 20 seconds.

Two-step real-time RT-PCR

i) Step 1: Reverse-transcription

Extracted RNA is reverse transcribed non-discriminately into cDNA using random primers. The cDNA synthesis reactions and cycling conditions are best performed using manufacturer's instructions for commercially available kits which have been extensively tested with a variety of RNA templates, including GC- and AU-rich targets and RNAse expressed at low levels.

ii) Step 2: Real-time PCR

The TaqMan real-time PCR assay uses forward primer 5'-ATG-AGG-CAG-GTG-TCG-GAG-G-3', reverse primer 5'-TGT-AGT-AGG-ACT-CTC-CCA-GCA-TCC and FAM-labelled probe 5'-6FAM-TAC-GCC-ATC-ATG-ATG-AGT-MGBNFQ-3'. Primers are used at a final concentration of 600 nM, and the final concentration of the probe is 200 nM. 2.5 µl of cDNA product is added to each 25 µl PCR reaction. Thermal cycling conditions are 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (Garver *et al.,* 2011).

A sample is negative if no Ct (threshold cycle) is recorded, while samples with a Ct are considered positive for VHSV. Cut-off value depends on the set-up in each laboratory.

4.4.2. Conventional RT-PCR

RNA isolation is done as in Section 4.4.1. Positive and negative controls should be run with each stage of the assays: extraction and RT-PCR. Due to the sensitive nature of PCR-based assays it is highly recommended that master mix, template addition and RT-PCR amplification occur in designated hoods or spatially separated areas.

A one-step RT-PCR should be performed as described by Kim *et al.* (2018) with 3F2R primer set: forward primers (3F, 5'-(GGG-ACA-GGA-ATG-ACC-ATG-AT-3') and reverse primer (2R, (5'-TCT-GTC-ACC-TTG-ATC–CCC-TCC-AG-3') targeting a 319 nt region in the nucleoprotein gene (positions 658–977).

The RT-PCR can be performed using, e.g. Qiagen OneStep RT-PCR System (Qiagen, Germany) or similar kit, according to the manufacturer's instructions. Briefly, the reaction mixture is adjusted to a final volume of 25 µl including 5 µl of extracted viral RNA, 5 µl 5 × One Step RT-PCR Buffer containing 12.5 mM $MgCl_2$ (final concentration 2.5 mM), 10 pM of each primer, and 1 µl of enzyme mix.

The following cycles are recommended: 50°C for 30 minutes, 95°C for 15 minutes, 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 60 seconds. Subsequently, the reaction is held at 68°C for 7 minutes.

4.4.3. Other nucleic acid amplification methods

To date, no other nucleic acid amplification method capable of universal VHSV detection has been sufficiently validated.

4.5. Amplicon sequencing

The VHSV genotype can be identified by sequencing the amplicon generated by the conventional RT-PCR using the 3F2R primer set (Kim *et al.,* 2018). Nucleotide sequencing of the glycoprotein gene is commonly used for identification of genetic strains and for epidemiological study and is recommended as one of the final steps for confirmatory diagnosis. There are several conventional RT-PCR assays available that amplify the central (669 nt) or full (1524 nt) glycoprotein gene coding sequence, but there are limited validation data. The glycoprotein gene can be amplified by conventional RT-PCR using the primer sets and concentrations listed in Table 4.2. The reverse transcription and subsequent PCR amplification can be done using a kit designed for that purpose according to the manufacturer's instructions.

Table 4.2. *Primer sets for the conventional RT-PCR, sequencing and phylogenetic analysis*

Primer	Sequence (5'–3')	Product size (bp)	Final primer concentration	Reference
GB+	GTC-GAA-GAA-GAG-ATA-GGC	1757	0.6 µM	Einer-Jensen *et al.,* 2004 Gudmundsdottir *et al.,* 2019
GB-	GTT-GGG-TCG-CCA-TGT-TTC-T		0.6 µM	
G330+	ACT-ACC-TAC-ACA-GAG-TGA-C	914	0.2 µM	Garver *et al*., 2013
G1243-	CAA-TTT-GTC-CCC-GAA-TAT-CAT		0.2 µM	
G422+	TCC-CGT-CAA-GAG-GCC-AC	669	0.2 µM	
G1179-	TTC-CAG-GTG-TTG-TTT-ACC-G		0.2 µM	

4.6. *In-situ* hybridisation

Not relevant in relation to primary diagnosis and surveillance of infection with VHSV.

4.7. Immunohistochemistry

Immunohistochemistry reveals VHSV-positive endothelial cells, primarily in the vascular system (Evensen *et al*., 1994). Specific polyclonal and monoclonal antibodies for immunohistochemistry are commercially available.

4.8. Bioassay

Not relevant in relation to primary diagnostics and surveillance of infection with VHSV.

4.9. Antibody- or antigen-based detection methods

Antibody- and antigen-based detection methods should not be used as a method of screening healthy populations.

4.9.1. Antigen enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for enzyme-linked immunosorbent assays (ELISAs) with appropriate dilutions of protein-A purified immunoglobulins (Ig) from rabbit anti sera against VHSV in carbonate buffer, pH 9.6 (50 µl well^{-1}).

ii) Incubate overnight at 4°C.

iii) Rinse in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST).

iv) Add 1% Triton X-100 to the virus suspension to be identified.

v) Dispense 50 µl well^{-1} of two- or four-step dilutions (in PBST containing 1% bovine serum albumin) of the virus to be identified and of VHSV control virus, as well as a negative control (e.g. infectious haematopoietic necrosis virus [IHNV]), and allow to react with the coated antibody to VHSV for 1 hour at 37°C.

vi) Rinse in PBST.

vii) Add to the wells monoclonal antibodies to VHSV N protein (IP5B11) 50 µl well^{-1}.

viii) Incubate for 1 hour at 37°C.

ix) Rinse in PBST.

x) Add to the wells (50 µl well^{-1}) horseradish peroxidase (HRP)-conjugated monoclonal anti-mouse antibodies.

xi) Incubate for 1 hour at 37°C.

xii) Rinse in PBST.

xiii) Visualise the reaction using TMB (3,3',5,5'-tetramethylbenzidine) and measure the absorbance at a wavelength of 450 nm.

The above ELISA version is given as an example. Other ELISA versions of demonstrated performance may be used instead.

For positive controls, use cell culture supernatant from cultures inoculated with known VHSV isolate.

For negative controls, use cell culture supernatant from same cell line inoculated with heterologous virus (e.g. IHNV) or from non-infected culture.

4.9.2. Indirect fluorescent antibody test (IFAT)

i) Prepare monolayers of cells in 2 cm^2 wells of cell culture plastic plates or on cover-slips to reach around 80% confluence, which is usually achieved within 24 hours of incubation at 22°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.

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

iv) Incubate at 15°C for 24 hours.

v) Remove the cell culture medium, rinse once with 0.01 M PBS, pH 7.2, then three times briefly with a cold mixture of acetone 30% and ethanol 70% (v/v) (stored at –20°C).

vi) Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 cm^2 of cell monolayer.

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

viii) Prepare a solution of purified VHSV antibody or serum in 0.01 M PBST, pH 7.2, at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Rehydrate the dried cell monolayers by using four rinsing steps with the PBST solution and remove this buffer completely after the last rinse.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur, e.g. by adding a piece of wet cotton in the humid chamber. The volume of solution to be used is 0.25 ml per 2 cm^2 well.

xi) Rinse four times with PBST as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)- or tetramethylrhodamine-5-(and-6-) isothiocyanate (TRITC)-conjugated antibody to the immunoglobulin used as the primary antibody and prepared according to the instructions of the supplier. These conjugated antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the cover-slips using, for example glycerol saline, pH 8.5 prior to microscopic observation.

xv) Examine under incident UV light using a microscope with ×10 eye pieces and ×20–40 objective lens having numerical aperture >0.65 and >1.3 respectively. Positive and negative controls must yield the expected results prior to any other observation.

Other IFAT or immunocytochemical (alkaline phosphatase or peroxidase) techniques of demonstrated performance may be used instead.

Always include positive control such as wells or coverslip with cells infected with a known VHSV isolate.

4.10. Other methods

4.10.1. Neutralisation test

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge it at 2000 ***g*** for 15 minutes at 4°C, or filter through a 0.45 µm (or 450 nm) pore membrane to remove cell debris.

ii) Dilute virus-containing medium from 10^{-2} to 10^{-4}.

iii) Mix aliquots (for example 200 µl) of each dilution with equal volumes of a VHSV antibody solution and, likewise, treat aliquots of each virus dilution with cell culture medium. The neutralising antibody [NAb] solution must have a 50% plaque reduction titre of at least 2000.

iv) In parallel, another neutralisation test must be performed against a homologous virus strain (positive neutralisation test).

v) If required, a similar neutralisation test may be performed using antibodies to IPNV.

vi) Incubate all the mixtures at 15°C for 1 hour.

vii) Transfer aliquots of each of the above mixtures on to 24–48 hour-old monolayers, overlaid with cell culture medium containing 10% FCS (inoculate two wells per dilution), and incubate at 15°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

viii) Check the cell cultures for the onset of CPE and read the result as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding the cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.

ix) The tested virus is identified as VHSV when CPE is prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the VHSV-specific antibody, whereas CPE is evident in all other cell cultures.

x) In the absence of any neutralisation by NAb to VHSV, it is mandatory to conduct an RT-PCR, an ELISA or IFAT, using the suspect sample. Some cases of antigenic drift of surface antigen have been observed, resulting in occasional failure of the neutralisation test using NAb to VHSV.

Other neutralisation tests of demonstrated performance may be used instead.

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

Virus isolation, real-time RT-PCR and conventional RT-PCR are the recommended tests for surveillance to demonstrate freedom of disease in apparently healthy population.

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 OIE 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 OIE 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, etc., 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 VHSV shall be suspected if at least one of the following criteria is met:

i) VHSV-typical CPE in cell cultures;

ii) A positive result from a real-time RT-PCR assay;

iii) A positive result from a conventional RT-PCR assay.

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with VHSV is considered to be confirmed if one or more of the following criteria is met:

i) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, IFAT or by conventional RT-PCR and sequencing of the amplicon;

ii) Detection of VHSV in tissue preparations by real-time RT-PCR, and by a conventional RT-PCR (targeting a non-overlapping region of the genome) and sequencing of the amplicon.

iii) Detection of VHSV in tissue preparations by immunohistochemistry, and by a conventional RT-PCR and sequencing of the amplicon.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.2 Clinically affected animals

No clinical signs are pathognomonic for infection with VHSV 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 VHSV shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with infection with VHSV as described in this chapter, with or without elevated mortality;

ii) Histopathological changes consistent with infection with VHSV as described in this chapter;

iii) A positive result from real-time RT-PCR;

iv) A positive result from a conventional RT-PCR;

v) A positive result by IFAT;

vi) VHSV-typical CPE in cell culture.

1 For example transboundary commodities.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with VHSV shall be confirmed if one or more of the following criteria is met:

i) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, IFAT or by conventional RT-PCR and sequencing of the amplicon;

ii) Detection of VHSV in tissue preparations by real-time RT-PCR, and by a conventional RT-PCR (targeting a non-overlapping region of the genome) and sequencing of the amplicon.

iii) Detection of VHSV in tissue preparations by immunohistochemistry, and by a conventional RT-PCR and sequencing of the amplicon.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with VHSV is provided in Table 6.3.1. This information can be used for the design of surveys for infection with VHSV, 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 two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

Table 6.3.1. *Diagnostic performance of tests recommended for surveillance or diagnosis*

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Cell culture	Surveillance	Experimentally infected fish	Kidney, heart and spleen	Rainbow trout	86 (84)	–	Real-time RT-PCR	Jonstrup *et al.,* 2013
Cell culture	Clinical diagnosis	Experimentally infected fish	Kidney	Atlantic salmon	100 (100)	94.4 (100)	Pseudo-gold standard*	Garver *et al.,* 2011
Real-time RT-PCR	Surveillance	Experimentally infected fish	Kidney	Atlantic salmon	93 (30)	100 (70)	Cell culture	Garver *et al.,* 2011
Real-time RT-PCR	Surveillance	Experimentally infected fish	Kidney, heart and spleen	Rainbow trout	90 (84)	100 (43)	Cell culture	Jonstrup *et al.,* 2013

* a compilation of 8 test results to evaluate both the real-time RT-PCR and virus isolation assay (Garver *et al,* 2011);
DSe = diagnostic sensitivity, DSp = diagnostic specificity; *n* = number of samples used in the study .

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NB: There are OIE Reference Laboratories for Infection with viral haemorrhagic septicaemia virus (see Table at the end of this *Aquatic Manual* or consult the OIE web site: https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the OIE Reference Laboratories for any further information on infection with viral haemorrhagic septicaemia virus

NB: FIRST ADOPTED IN 1995 AS VIRAL HAEMORRHAGIC SEPTICAEMIA.
MOST RECENT UPDATES ADOPTED IN 2021.