

## VIRAL HAEMORRHAGIC SEPTICAEMIA

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### 1. Scope<sup>1</sup>

For the purpose of this chapter, viral haemorrhagic septicaemia (VHS) is considered to be a disease caused by infection with viral haemorrhagic septicaemia virus (VHSV, synonym: Egtved virus).

VHS is a disease of farmed rainbow trout, farmed turbot, farmed Japanese flounder as well as a broad range of wild freshwater and marine species (European Food Safety Authority, 2008; Meyers & Winton, 1995; Skall et al., 2005) caused by VHSV, a virus belonging to the genus *Novirhabdovirus*, within the family *Rhabdoviridae* (Walker et al., 2000). All VHS virus isolates can be identified by immunochemical tests using the monoclonal antibody IP5B11 (Lorenzen et al., 1988).

Diseased fish may display nonspecific clinical signs in the early stages of infection, including rapid onset of mortality (which can reach up to 100% in fry), lethargy, darkening of the skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of the fins, gills, eyes and skin, and a distended abdomen due to oedema in the peritoneal cavity. In the chronic state of infection, affected fish do not in general exhibit external signs. VHS can also occur in a nervous form, characterised by severe abnormal swimming behaviour, such as constant flashing and/or spiralling. Corroborative diagnostic criteria are summarised in Section 7 of this chapter.

### 2. Disease information

#### 2.1. Agent factors

##### 2.1.1. Aetiological agent, agent strains

The aetiological agent of VHS is a rhabdovirus (VHSV) belonging to the genus *Novirhabdovirus*, within the family *Rhabdoviridae*, which also includes infectious haematopoietic necrosis virus (IHNV) and the Hirame rhabdovirus of the Japanese flounder (*Paralichthys olivaceus*). Virions are bullet-shaped (approximately 70 nm in diameter and 180 nm in length), contain a negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides and possess 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).

Rainbow trout, in which VHSV can cause severe disease outbreaks, are traditional hosts of the virus, but many other freshwater and marine fish species are also susceptible to the disease. Natural outbreaks have occurred in farmed turbot (Ross et al., 1994; Schlotfeldt et al., 1991) and in both farmed and wild Japanese flounder (Isshiki et al., 2001; Takano et al., 2000). Mortality from natural infection in free-living marine fish species has been observed along the Pacific coast of North America (Meyers et al., 1999; Traxler et al., 1999). In addition, isolates from Pacific herring have been shown to be pathogenic for Pacific herring under experimental conditions (Kocan et al., 1997). For a review see Skall et al., 2005. Likewise, VHS outbreaks have occurred in wild, freshwater species in the Great Lakes (Elsayed et al., 2006; Groocock et al., 2007; Lumsden et al., 2007).

The large host range and the significant differences in pathogenicity in different host species can cause problems for VHS control programmes, which are based mainly on the protection of the significant rainbow trout production industry. The main problem is whether the finding of marine VHSV in free-living fish in an approved VHSV-free area should lead to the withdrawal of that status. However, it has been observed in a large part of Europe that VHS in free-living fish in the marine environment affects an approved VHS-free status in only very few cases. Nevertheless, a recent outbreak in rainbow trout in Norway was caused by genotype III VHSV, a marine genotype until then not considered to be pathogenic for rainbow trout (Dale et al., 2009), indicating that marine strains are not without significance to the farmed rainbow trout industry.

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1 NB: Version adopted by the World Assembly of Delegates of the OIE in May 2012.

The monoclonal antibody (MAb) IP5B11 (Lorenzen et al., 1988) reacts with all VHSV isolates of all known geno- and serotypes.

Most polyclonal antibodies raised against VHSV type I (DK-F1) cross react with all known VHSV isolates in the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assays (ELISA). In the neutralisation test, however, VHSV can be divided into three subgroups based on the neutralisation pattern towards a panel of four neutralising MAbs and one polyclonal antibody (Olesen et al., 1993). VHSV thus shares several antigenic epitopes, although sero-grouping does not correlate with genotypes identified using nucleic acid sequence analysis. MAbs specifically reacting with different groups of VHSV, e.g. the American/Japanese genogroup IVa and genogroup Ib isolates, have been developed (Ito et al., 2010; Ito et al., manuscript in prep.).

The most discriminatory way of typing VHSV is by nucleic acid sequencing. Sequence comparisons of many VHSV isolates by several laboratories have shown that genetic differences appear to be related more to geographical location than to year of isolation or host species (Skall et al., 2005). Four major genotypes have been grouped, based on sequencing of full-length and/or truncated genes from the N-gene (Einer-Jensen et al., 2005; Snow et al., 1999; Snow et al., 2004), G-gene (Einer-Jensen et al., 2004; Einer-Jensen et al., 2005) and NV-gene (Einer-Jensen et al., 2005):

Genotype I: Several sublineages (Ia-Ie) containing European freshwater VHSV isolates, isolates from the Black Sea area and a group of marine isolates from the Baltic Sea, Kattegat, Skagerrak, the North Sea and the English Channel. Genotype Ib isolates were recently recovered as far north as latitude 70°N close to Nordkap in Norway (www.fishpathogens.eu, report no. 2902).

Genotype II: A group of isolates from the Baltic Sea

Genotype III: Isolates from the North Atlantic Sea (from the Flemish Cap (López-Vázquez et al., 2006) to the Norwegian coast (Dale et al., 2009), the North Sea, Skagerrak and Kattegat.

Genotype IV: North American and Japanese/Korean isolates (two sublineages IVa and IVb [Elsayed et al., 2006]).

Genotype I is divided into several sublineages, where the marine isolates from wild fish fall into sublineage Ib. The best resolution of genotype I sublineages is obtained when analysing the full-length G-gene (Einer-Jensen et al., 2005).

As genotype I comprises VHSV isolated from wild marine fish, as well as isolates causing mortality in rainbow trout from continental Europe, a relationship between freshwater and marine types was suggested (Skall et al., 2005).

All the Japanese and other Asian isolates but one fall into the North American IVa genotype. The remaining isolate falls into the traditional European genotype Ib (Nishizawa et al., 2002). This isolate is considered to have been introduced from outside Japan.

In North America, at least two sublineages are found: genotype IVa on the Pacific coast and genotype IVb on the Atlantic coast and in the Great Lakes region.

### 2.1.2. Survival 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). 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). The virus can last a longer time if organic materials are added to the water, such as ovarian fluids or blood products such as bovine serum. 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 other studies 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).

Freezing VHSV-infected fish at commercial freezing temperatures then thawing the fish will not completely kill the virus, but will reduce infectivity or virus titres of 90% or more (Arkush et al., 2006). These authors noted that the remaining infectious virus remained in the fish tissue and was not lost in the water thawed from the frozen fish.

### 2.1.3. Stability of the agent (effective inactivation methods)

VHSV is sensitive to a number of common disinfectants. For reviews see Bovo et al., 2005b; Wolf, 1988.

## 2.2. Host factors

The reservoirs of VHSV are clinically infected fish as well as covert carriers among cultured, feral or wild fish. Several factors influence susceptibility to the disease VHS. In rainbow trout there is genetic variability for susceptibility (Henryon et al., 2002a; Henryon et al., 2002b), and the age of the fish appears to be of some importance - the younger the fish the higher the susceptibility. In general, older fish experiencing high VHS mortality have never been in contact with the disease before.

### 2.2.1. Susceptible host species

During the past two decades VHSV has been isolated from an increasing number of marine and freshwater fish species (see Tables 2.1 and 2.2.). (Table 2.1 provides a list of species for which there is conclusive scientific evidence of susceptibility and Table 2.2 provides a list of species for which there is some evidence of susceptibility.) It is likely that VHSV is endemic in fish populations in large areas of the temperate Northern Hemisphere. So far, VHSV has been isolated from approximately 80 different fish species throughout the Northern Hemisphere, including North America, Asia and Europe. A number of species have been shown to be susceptible to VHSV under experimental conditions. The number of possible host species is increasing with increasing monitoring efforts. However, the most susceptible farmed fish species is rainbow trout genotype Ia, although VHS is also reported to cause mortality in farmed turbot and Japanese flounder. Among wild fish, severe die-offs have been observed in recent years in the Great Lakes region of the USA and Canada involving at least 28 freshwater fish species. All VHSV isolates from these outbreaks belong to genotype IVb (United States Department of Agriculture (USDA), 2008; Thompson et al., 2011).

**Table 2.1.** Fish species for which there is conclusive evidence of susceptibility (European Food Safety Authority [EFSA], 2008)

Susceptible according to European Food Safety Authority (EFSA) rules			
Order	Family	Common name	Latin name
Salmoniformes (salmon)	Salmonidae (salmonids)	Rainbow trout / Steelhead trout	<i>Oncorhynchus mykiss</i>
		Chinook salmon	<i>Oncorhynchus tshawytscha</i>
		Coho salmon	<i>Oncorhynchus kisutch</i>
		Atlantic salmon	<i>Salmo salar</i>
		Brown trout	<i>Salmo trutta</i>
		Grayling	<i>Thymallus thymallus</i>
		Whitefish <sup>a</sup>	<i>Coregonus lavaretus</i>
		Whitefish	<i>Coregonus</i> spp.
		— <sup>a</sup>	<i>O. mykiss</i> × <i>O. kisutch</i>
		— <sup>a</sup>	<i>O. mykiss</i> × <i>S. fontinalis</i> triploid
— <sup>a</sup>	<i>O. mykiss</i> × <i>S. alpinus</i> triploid		
Esociformes	Esocidae	Muskellunge	<i>Esox masquinongy</i>
		Northern pike	<i>Esox lucius</i>
Clupeiformes	Clupeidae	Atlantic herring	<i>Clupea harengus</i>
		Pacific herring	<i>Clupea pallasii</i>
		South American pilchard	<i>Sardinops sagax</i>
		European sprat	<i>Sprattus sprattus</i>

**Table 2.1.** Fish species for which there is conclusive evidence of susceptibility (European Food Safety Authority [EFSA], 2008)

Susceptible according to European Food Safety Authority (EFSA) rules			
Order	Family	Common name	Latin name
Gadiformes (cod)	Gadidae	Atlantic cod	<i>Gadus morhua</i>
		Poor cod	<i>Trisopterus minutus</i>
		Whiting	<i>Merlangius merlangus</i>
		Blue whiting	<i>Micromesistius poutassou</i>
		Norway pout	<i>Trisopterus esmarkii</i>
		Alaska Pollock	<i>Theragra chalcogramma</i>
	Lotidae (hakes and burbot)	Fourbeard rockling	<i>Enchelyopus cimbrius</i>
		Burbot	<i>Lota lota</i>
Merlucciidae	(North) Pacific hake	<i>Merluccius productus</i>	
Pleuronectiformes (flatfish)	Pleuronectidae	Dab	<i>Limanda limanda</i>
		Flounder	<i>Platichthys flesus</i>
		European plaice	<i>Pleuronectes platessa</i>
		Greenland halibut	<i>Reinhardtius hippoglossoides</i>
		Atlantic halibut <sup>a</sup>	<i>Hippoglossus hippoglossus</i> <sup>b</sup>
	Scophthalmidae	Turbot	<i>Scophthalmus maximus</i>
	Paralichthyidae	Japanese flounder	<i>Paralichthys olivaceus</i>
Osmeriformes	Argentinidae	Lesser argentine	<i>Argentina sphyraena</i>
	Osmeridae (smelt)	Surf smelt	<i>Hypomesus pretiosus</i>
Perciformes (perch-like)	Ammodytidae	Pacific sand lance	<i>Ammodytes hexapterus</i>
		Sand eel	<i>Ammodytes</i> spp.
		Pacific sand eel	<i>Ammodytes personatus</i>
	Gobiidae	Sand goby	<i>Pomatoschistus minutus</i>
		Round goby	<i>Neogobius melanostomus</i>
	Embiotocidae	Shiner perch	<i>Cymatogaster aggregata</i>
	Sciaenidae	Freshwater drum	<i>Aplodinotus grunniens</i>
	Scombridae	Chub mackerel, Pacific mackerel	<i>Scomber japonicus</i>
	Percidae(perch)	Yellow perch	<i>Perca flavescens</i> <sup>c</sup>
Moronidae(temperate bass)	European seabass <sup>a</sup>	<i>Dicentrarchus labrax</i>	
Gasterosteiformes	Gasterosteidae	Three-spined stickleback	<i>Gasterosteus aculeatus</i>
Cypriniformes (carp)	Cyprinidae(minnows or carp)	Fathead minnow <sup>a</sup>	<i>Pimephales promelas</i> <sup>d</sup>
Petromyzontiformes (lamprey)	Petromyzontidae (lamprey)	European river lamprey	<i>Lampetra fluviatilis</i> <sup>e</sup>

a. Infection trial, immersion;

b. Skall et al., 2005;

c. Kane-Sutton et al., 2010;

d. Al-Hussinee et al., 2010;

e. Gadd et al., 2010.

**Table 2.2.** Fish species for which there is some evidence of susceptibility (EFSA, 2008)

Insufficient data to state susceptibility			
Order	Family	Common name	Latin name
Salmoniformes (salmon)	Salmonidae (salmonids)	Chum salmon <sup>a</sup>	<i>Oncorhynchus keta</i>
		Sockeye salmon <sup>a</sup>	<i>Oncorhynchus nerka</i>
		Lake trout <sup>b c</sup>	<i>Salvelinus namaycush</i> <sup>d</sup>
		Brook trout <sup>bc</sup>	<i>Salvelinus fontinalis</i> <sup>d</sup>
		Lake whitefish	<i>Coregonus clupeaformis</i>
		Golden trout <sup>c</sup>	<i>Oncorhynchus aguabonita</i>
		Artic char <sup>b</sup>	<i>Salvelinus alpinus</i>
		Splake <sup>c</sup>	<i>Salvelinus namaycush</i> x <i>Salvelinus fontinalis</i> <sup>d</sup>
		_b	<i>O. mykiss</i> x <i>S. namaycush</i>
		_b	<i>O. mykiss</i> x <i>O. kisutch</i> triploid
Clupeiformes	Clupeidae	American gizzard shad	<i>Dorosoma cepedianum</i>
Gadiformes (cod)	Gadidae	Pacific cod	<i>Gadus macrocephalus</i>
		Haddock	<i>Melanogrammus aeglefinus</i>
		Pacific tomcod	<i>Microgadus proximus</i>
	Lotidae(hakes and burbot)	Burbot	<i>Lota lota</i>
Siluriformes (catfish)	Ictaluridae (North American freshwater catfish)	Brown bullhead	<i>Ictalurus nebulosus</i>
		Channel catfish	<i>Ictalurus punctatus</i>
Osmeriformes	Osmeridae (smelt)	Eulachon	<i>Thaleichthys pacificus</i>
Perciformes (perch-like)	Centrarchidae (sunfish)	Largemouth bass	<i>Micropterus salmoides</i>
		Largemouth bass <sup>c</sup>	<i>Micropterus salmoides</i> <sup>d</sup>
		Smallmouth bass	<i>Micropterus dolomieu</i>
		Bluegill	<i>Lepomis macrochirus</i>
		Pumpkinseed	<i>Lepomis gibbosus</i>
		Black crappie	<i>Pomoxis nigromaculatus</i>
		Rock bass	<i>Ambloplites rupestris</i>
	Percidae (perch)	Walleye	<i>Sander vitreus</i>
		European perch	<i>Perca fluviatilis</i>
	Moronidae (temperate bass)	White bass	<i>Morone chrysops</i>
		Striped bass	<i>Morone saxatilis</i>
		White perch	<i>Morone americana</i>
		Black porgy <sup>c</sup>	<i>Acanthopagrus schlegelii</i>
		Red seabream <sup>c</sup>	<i>Pagrus major</i>
	Sparidae	Gilthead seabream	<i>Sparus aurata</i>

**Table 2.2.** Fish species for which there is some evidence of susceptibility (EFSA, 2008)

Insufficient data to state susceptibility			
Order	Family	Common name	Latin name
Perciformes (perch-like)	<i>Serranidae</i>	Hong Kong grouper <sup>c</sup>	<i>Epinephelus akaara</i>
	<i>Carangidae</i>	Japanese amberjack <sup>c</sup>	<i>Seriola quinqueradiata</i>
	<i>Sciaenidae</i>	Yellow croacker <sup>a</sup>	<i>Larimichthys polyactis</i> <sup>e</sup>
	<i>Sparidae</i>	Yellowback seabream <sup>a</sup>	<i>Dentex tumifrons</i> <sup>e</sup>
	<i>Trichiuridae</i>	Largehead hairtail <sup>a</sup>	<i>Trichiurus lepturus</i> <sup>e</sup>
	<i>Stromateidae</i>	Silver pomfret, butter fish	<i>Pampus argentus</i> <sup>e</sup>
Scorpaeniformes (scorpionfish and flatheads)	<i>Anoplopomatidae</i> (sablefish)	Sablefish	<i>Anoplopoma fimbria</i>
	<i>Sebastidae</i> (Rockfish, rockcod and thornyheads)	Black rockfish, Mebaru (Japanese)	<i>Sebastes inermis</i>
		Schlegel's black rockfish <sup>c</sup>	<i>Sebastes schlegelii</i>
	<i>Liparidae</i>	Cubed snailfish <sup>a</sup>	<i>Liparis tessellatus</i> <sup>e</sup>
<i>Scorpaenidae</i>	Izu scorpionfish, sting fish <sup>a</sup>	<i>Scorpaena izensis</i> <sup>e</sup>	
Anguilliformes	<i>Anguillidae</i>	European eel	<i>Anguilla anguilla</i>
		American eel <sup>a</sup>	<i>Anguilla rostrata</i> <sup>f</sup>
Cyprinodontiformes	<i>Fundulidae</i>	Mummichog	<i>Fundulus heteroclitus</i>
Gasterosteiformes	<i>Aulorhynchidae</i>	Tube-snout	<i>Aulorhynchus flavidus</i>
Cypriniformes (carp)	<i>Catostomidae</i>	Silver redbhorse	<i>Moxostoma anisurum</i>
		Shorthead redbhorse	<i>Moxostoma macrolepidotum</i>
	<i>Cyprinidae</i> (minnows or carp)	–	<i>Barbus graellsii</i>
		Bluntnose minnow	<i>Pimephales notatus</i>
		Emerald shiner	<i>Notropis atherinoides</i>
		Spottail shiner	<i>Notropis hudsonius</i>
		Iberian nase	<i>Chondrostoma polylepis</i>
		Zebra danio <sup>c</sup>	<i>Danio rerio</i>
Goldfish <sup>b</sup>	<i>Carassius auratus</i>		
Percopsiformes (trout-perch, pirate perch and cavefish)	<i>Percopsidae</i> (trout-perch)	Trout-perch	<i>Percopsis omiscomaycus</i>
Pleuronectiformes (flatfish)	<i>Soleidae</i>	Senegalese sole	<i>Solea senegalensis</i>
		Senegalese sole <sup>bc</sup>	<i>Solea senegalensis</i> <sup>g</sup>
	<i>Pleuronectidae</i>	Marbled flounder <sup>c</sup>	<i>Pleuronectes yokohamae</i>
		English sole	<i>Parophrys vetula</i>
		Blackfin flounder, Korean flounder <sup>a</sup>	<i>Glyptocephalus stelleri</i> <sup>e</sup>

**Table 2.2.** Fish species for which there is some evidence of susceptibility (EFSA, 2008)

Insufficient data to state susceptibility			
Order	Family	Common name	Latin name
Mugiliformes	<i>Mugilidae</i>	Flathead grey mullet, striped mullet	<i>Mugil cephalus</i> <sup>d</sup>
		Flathead grey mullet, striped mullet <sup>a</sup>	<i>Mugil cephalus</i> <sup>e</sup>
Ophidiiformes	<i>Ophidiidae</i>	Armoured cusk, Armoured weaselfish <sup>a</sup>	<i>Hoplobrotula armata</i> <sup>e</sup>
Carcharhiniformes	<i>Scyliorhinidae</i>	Cloudy catshark <sup>a</sup>	<i>Scyliorhinus torozame</i> <sup>e</sup>
Non-fish		Leech	<i>Myzobdella lugubris</i> <sup>h</sup>
Non-fish		Crustacean	<i>Diporeia</i> spp. <sup>i</sup>

- a. Reverse-transcription polymerase chain reaction (RT-PCR) only;  
b. Infection trial, immersion;  
c. Infection trial, IP injection;  
d. Kim & Faisal, 2010;  
e. Lee et al., 2007;  
f. Al-Hussinee et al., 2011;  
g. López-Vázquez et al., 2011;  
h. Faisal & Schulz, 2009;  
i. Faisal & Winters, 2011.

### 2.2.2. Susceptible stages of the host

Infection with VHSV may cause disease and mortality in all life stages of susceptible fish. Infection may result in the development of protective immunity in endemic areas; the disease is therefore more abundant in populations of young, not previously infected fish. VHSV is not known to infect fish eggs.

### 2.2.3. Species or subpopulation predilection (probability of detection)

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. Highest prevalence of virus was found in shoaling fish, such as herring, sprat, Norway pout, etc. (Skall et al., 2005).

### 2.2.4. Target organs and infected tissue

In septic stages of the disease, the virus is abundant in all tissues including skin and muscles. Target organs are 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. Persistent infection with lifelong carriers

Some survivors of epizootics will become long-term carriers of the virus.

### 2.2.6. Vectors

In view of the large number of susceptible species, the virus may be presumed to be able to multiply in a sufficiently broad range of host to preclude the need for vectors. In many fish species, clinical signs have, however, never been observed in infected individuals.

VHSV has been isolated from leech, *Myzobdella lugubris*, and from *Diporeia* spp. in the Great Lakes, North America. Whether the leech and the shrimp-like *Diporeia* can transmit VHSV from one fish to another is unknown at present (Faisal & Schulz, 2009; Faisal & Winters, 2011).

VHSV can be transferred by piscivorous birds as external mechanical vectors (Olesen & Vestergård Jørgensen, 1982; Peters & Neukirch, 1986).

### 2.2.7. Known or suspected wild aquatic animal carriers

See Tables 2.1 and 2.2 for species from which VHSV has been isolated. Currently, there is insufficient scientific evidence to support the listing of all the species from the Great Lakes as being susceptible to VHSV (European Food Safety Authority, 2008).

## 2.3. Disease pattern

### 2.3.1. Transmission mechanisms

Knowledge of the mechanism of virus transmission has come mainly from studies of rainbow trout VHSV isolates from Europe; these have shown transmission to be horizontal through contact with other infected fish or contaminated water, etc. Virus is shed from infected fish via the urine (Wolf, 1988) and reproductive fluids. Transmission readily occurs in the temperature range 1–15°C but can occur up to 20°C. Incubation time is dependent on temperature and dose; it is 5–12 days at higher temperatures.

During and immediately following an outbreak, virus can be isolated readily in cell culture (Wolf, 1988). Kidney, heart and spleen tissues yield the highest viral titres.

In carrier (clinically healthy) fish, detection of VHSV is more problematic (Skall et al., 2005). VHSV will grow in a range of fish cell lines, with the BF-2 cell line being the most sensitive to infection by freshwater European strains (Skall et al., 2005). Cell susceptibility is ranked in the order BF-2, FHM, RTG-2, and EPC (Lorenzen et al., 1999), but other fish cell lines, such as CHSE-214 and SSN-1, are also susceptible. Susceptibility of a cell line to infection will depend on a range of parameters, including cell-line lineage and viral strain differences; it thus appears that the EPC cell line may be more susceptible to VHSV genotype IV isolates than to type I II or III isolates (Skall et al., 2005).

The carrier status of VHSV in freshwater fish species is well established (Enzmann & Konrad, 1985; Jørgensen, 1982). The virological status of such carriers will be dependent on a range of parameters including the length of time following initial exposure and geographical proximity to fish-farm outlets. Since the discovery of VHSV strains in marine species, there have been a number of studies involving extensive sampling of a broad range of fish species from the coastal waters of continental Europe (Skall et al., 2005), the UK (Skall et al., 2005), North America (Hedrick et al., 2003) and Asia (Kim & Park, 2004; Takano et al., 2000). In these studies, samples were analysed for the presence of virus by inoculation onto fish cell lines. In some studies, fish samples were pooled and therefore determination of precise prevalence was difficult. Nevertheless, based on virus isolation in cell culture and irrespective of the fish species examined, the prevalence of VHSV in the marine fish species sampled in these studies was in the range of 0.0–16.7% (95% confidence interval 8.7–27.5%) (Skall et al., 2005).

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 a modest accumulated mortality.

Low water temperatures (1–5°C) generally result in an extended disease course with low daily mortality but high accumulated mortality. VHS outbreaks occur during all seasons, but are most common in spring when water temperatures are rising or fluctuating. For more detailed reviews of the condition, see reviews by Wolf, 1988 and Smail & Snow, 2011.

Transmission primarily occurs horizontally through water, with excretion of virus in the urine (Smail & Snow, 2011). Studies using bioluminescence imaging of live trout infected with recombinant virulent IHNV, a virus very similar to VHSV, carrying a reporter gene, elegantly illustrated that virus yield on the fish skin was very high (Bremont, 2005). This remains to be examined for VHS-infected fish, but the direct excretion of virus from the skin may be a source of virus spread (Smail & Snow, 2011).

There are no indications or evidence of true vertical transmission of VHSV (Bovo et al., 2005a).

### 2.3.2. Prevalence

Until the late 1980s, VHS was considered to be restricted to farmed rainbow trout in continental Europe, with the occasional isolation from a restricted number of other freshwater fish species (e.g. brown trout, pike [Meier & Jørgensen, 1980; Schlotfeldt & Ahne, 1988]) with Scandinavia (except Denmark), Great Britain, and Ireland considered as VHS free. With the detection and isolation of VHSV from Pacific salmon off the Pacific North American coast in the late 1980s, subsequent studies have demonstrated that VHSV occurs in numerous farmed and wild fish species along the Pacific and Atlantic North American coast (Skall et al., 2005), in the Great Lakes area of North America (Thompson et al., 2011) the seas around the UK (Skall et al., 2005), the

Baltic Sea, Skagerrak and Kattegat (Skall et al., 2005), in the waters around Japan (Skall et al., 2005), and in the Black Sea area, with a distinct genotype Ie (Nishizawa et al., 2006).

### **2.3.3. Geographical distribution**

During the past two decades, VHSV has been isolated from wild fish throughout the temperate area of the Northern Hemisphere, both in fresh- and marine waters (Skall et al., 2005). VHS outbreaks in farmed rainbow trout have, however, only been experienced in Europe, where it is still considered to be one of the most serious viral diseases in aquaculture. In America, VHS primarily causes mortality in wild fish (Meyers & Winton, 1995; Skall et al., 2005; United States Department of Agriculture (USDA), 2008). In Asia, there have been reports of clinical outbreaks in farmed Japanese flounder as well as isolations from wild fish species (Lee et al., 2007; Skall et al., 2005).

### **2.3.4. Mortality and morbidity**

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%. Immersion infection trials also induced up to 100% mortality in Pacific herring when infected with genotype IVa (Skall et al., 2005).

### **2.3.5. Environmental factors**

VHS outbreaks have been reported in both freshwater and seawater environments with salinities up to 36 parts per thousand (ppt) and at temperatures ranging from 2 to 20°C. Most disease outbreaks are observed in spring when temperatures are fluctuating.

Laboratory studies have shown that the temperature range of VHSV genotype IVb appears to be the same as genotype I, with an optimum of 9–12°C and an upper limit of 18–20°C (Goodwin & Merry, 2011).

## **2.4. Control and prevention**

In the absence of anti-viral treatments, control methods for VHS currently lie in official health surveillance schemes coupled with control measures. Examples of practices that have been successful in reducing the number of infected farms in an endemic area and preventing reinfection, such as stamping-out and fallowing procedures, have been reviewed previously (Olesen, 1998; Olesen & Korsholm, 1997). Successful eradication of acute disease outbreaks were recently experienced in the UK in 2006 (Stone et al., 2008) and Norway in 2007 (Dale et al., 2009) as outbreaks have not been observed since, and Denmark, which had more than 400 endemically infected farms freed itself from VHS after 45 years of surveillance and control experiencing the last disease outbreak in February 2009 (Manuscript in preparation).

### **2.4.1. Vaccination**

Although research on vaccine development for VHS has been ongoing for more than three 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. However, the latter have proven to be very promising inducing good protection from VHS. For a review see Lorenzen & Lapatra, 2005.

### **2.4.2. Chemotherapy**

No therapies are currently available.

### **2.4.3. Immunostimulation**

Several immunostimulants, such as yeast-derived beta-glucans, IL-1 $\beta$ -derived peptides, and probiotics have been assessed for enhancing protection against VHS (Peddie et al., 2003). Several authors report positive effects, but no immunostimulant directed specifically at enhanced resistance to VHS is available.

#### 2.4.4. Resistance breeding

Additive genetic variation in rainbow trout has been detected for resistance to VHS (Dorson et al., 1995; Henryon et al., 2002a; Henryon et al., 2002b). In a study by Henryon et al., 2005, the heritability of resistance to VHS was 0.11 for time to death on a logarithmic time-scale. It has thus been shown that there is a good potential for resistance breeding. However, no resistant rainbow trout strains are yet commercially available.

#### 2.4.5. Restocking with resistant species

In some farms in Denmark, with recurrent high mortalities caused by VHS, restocking with more resistant species such as brown trout (*Salmo trutta*) and pike-perch (*Sander lucioperca*) has been attempted (H. Korsholm, pers. comm.).

#### 2.4.6. Disinfection of eggs and larvae

Disinfection of eyed and green eggs is an efficient and cost-effective preventative measure for stopping the spread of the disease (for detailed procedures see Bovo et al., 2005b).

#### 2.4.7. General husbandry practices

Poor water quality, high fish density, high feeding rate, other diseases such as proliferative kidney disease (PKD), ichthyophthiriasis, bacterial kidney disease (BKD), etc. can influence the course and severity of disease. In general, an increase in temperature, restricted feeding, reduced fish density and restricted handling may reduce mortality. In endemically infected farms, stocking with naïve alevins is usually done at as high water temperatures as possible.

### 3. Sampling

#### 3.1. Selection of 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:

- For genotype I: in farms with salmonids, if rainbow trout are present, only 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 Tables 2.1 and 2.2. However, the species should be proportionally represented in the sample. For other genotypes: species of known susceptibility to the genotype in question should be sampled. 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 risk status).
- If more than one water source is used for fish production, fish from all water sources should be included in the sample.
- 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.

#### 3.2. Preservation of samples for submission

Before shipment or transfer to the laboratory, parts of the organs to be examined must be removed from the fish with sterile dissection instruments and transferred to sterile plastic tubes containing transport medium, i.e. cell culture medium with 10% fetal calf serum (FCS) and antibiotics. The combination of 200 International Units (IU) penicillin, 200 µg streptomycin, and 200 µg kanamycin per ml are recommended, although other antibiotics of proven efficiency may also be used.

### 3.3. Pooling of samples

Ovarian fluid or organ pieces from a maximum of ten fish may be collected in one sterile tube containing at least 4 ml of transport medium and this represents one pooled sample. The tissue in each sample should weigh a minimum of 0.5 g. The tubes should be placed in insulated containers (for instance, thick-walled polystyrene boxes) together with sufficient ice or 'freezer blocks' to ensure chilling of the samples during transportation to the laboratory. Freezing must be avoided. The temperature of a sample during transit should never exceed 10°C and ice should still be present in the transport box at receipt or one or more freezer blocks must still be partly or completely frozen. Virological examination must be started as soon as possible and not later than 48 hours after collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after collection of the material, provided that the material to be examined is protected by transport medium and that the temperature requirements during transportation are fulfilled.

Whole fish may be sent to the laboratory if the temperature requirements during transportation can be fulfilled. Whole fish may be wrapped in paper with absorptive capacity and must be shipped in a plastic bag, chilled as mentioned above. Live fish can also be shipped. All packaging and labelling must be performed in accordance with present national and international transport regulations, as appropriate.

### 3.4. Best organs or tissues

The optimal tissue material to be examined is spleen, anterior kidney, and either heart or encephalon. In some cases, ovarian fluid and milt must be examined.

In case of small fry, whole fish less than 4 cm long can be minced with sterile scissors or a scalpel after removal of the body behind the gut opening. If a sample consists of whole fish with a body length between 4 cm and 6 cm, the viscera including kidney should be collected.

### 3.5. Samples/tissues that are not suitable

VHSV is very sensitive to enzymatic degradation, therefore sampling tissues with high enzymatic activities such as viscera and liver should be avoided.

## 4. Diagnostic methods

### 4.1. Field diagnostic methods

#### 4.1.1. Clinical signs

The occurrence of the following signs should lead to extended clinical examination for VHS: rapid onset of mortality, lethargy, darkening of the skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of the fins, gills, eyes and skin, abnormal swimming such as flashing and spiralling, and a distended abdomen due to oedema in the peritoneal cavity.

#### 4.1.2. Behavioural changes

The nervous form of the disease is characterised by severe abnormal swimming behaviour, such as constant flashing and spiralling, because of the tropism of the virus for the brain. Unlike fish with bacterial septicaemia, VHS-infected fish will tend not to escape when being netted.

### 4.2. Clinical methods

#### 4.2.1. 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 VHS infection. The kidney is dark red in the acute phase, but can demonstrate severe necrosis in moribund fish. The spleen is moderately swollen. The liver is often pale and mottled. The gastrointestinal tract, especially the hind gut, is pale and devoid of food.

#### 4.2.2. Clinical chemistry

The red blood cell level is very low in the acute phase of VHS and the blood appears light red and transparent.

### 4.2.3. Microscopic pathology

Immunohistochemistry reveals VHSV-positive endothelial cells, primarily in the vascular system (Evensen et al., 1994). 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 site of infection, erythrocytes can accumulate in the skeletal muscle bundles and fibres without causing damage to the muscle *per se*.

### 4.2.4. Electron microscopy/cytopathology

VHSV is a typical bullet-shaped rhabdovirus, 60–75 nm in diameter and 180–240 nm in length. Ultrastructural aspects of the development of viral infection in cell culture have been described previously (Baroni et al., 1982).

## 4.3. Agent detection and identification methods

The standard surveillance method (to detect carrier fish) for VHS is based on direct methods, i.e. the isolation of VHSV in cell culture followed by identification using antibody-based methods (IFAT, ELISA) or nucleic acid-based methods (e.g. reverse-transcription polymerase chain reaction [RT-PCR]). As the direct immunological demonstration of VHSV antigen in infected fish tissues (by antibody-binding assay) has low sensitivity, it can only be used when VHS infection is suspected (based on clinical signs, epidemiological data and histopathology). A recently published validated real-time RT-PCR for direct identification of the VHSV genome in fish tissue was found to have sensitivity and specificity values very similar to cell culture methods followed by identification (Garver et al., 2011; Jonstrup et al., 2013). The technique has the potential to be used in direct surveillance programmes for obtaining approved VHS-free status, should, in the future, the OIE Member Countries approve its use for this purpose.

### 4.3.1. Direct detection methods

#### 4.3.1.1. Microscopic methods

The kidney and liver are prime targets and examination of histological sections from diseased fish reveals degeneration and necrosis of haematopoietic tissues of the kidney (and the spleen) with focal degeneration and necrosis of the liver. Sections of the skeletal muscle may show many foci of red blood cells, while the muscle fibres remain undamaged.

#### 4.3.1.2. Agent isolation and identification

##### 4.3.1.2.1. Cell culture/artificial media

Isolation of VHS virus in cultures of a number of established fish cell lines is well-documented (Lorenzen et al., 1999; Olesen & Vestergård Jørgensen, 1992). While inoculation of fish cell lines with fish tissues processed for virus isolation is considered the 'Gold' standard for surveillance programmes (for detecting carrier fish) with respect to sensitivity, the precise sensitivity of the procedure is unknown. Infected fish material suitable for virological examination is dependent on fish size. Thus, whole alevin (body length <4 cm), viscera including kidney (4 cm < body length < 6 cm) or, for larger size fish, kidney, spleen, heart and encephalon, and ovarian fluid from broodfish at the time of spawning are suitable samples.

The fish cell line BF-2 is recommended. Alternatively, EPC or FHM cells may be used (Lorenzen et al., 1999; Olesen & Vestergård Jørgensen, 1992; United States Department of the Interior, 2007). For the Genotypes I, II and III EPC cells are, in general, less susceptible than BF-2 (Lorenzen et al., 1999; Olesen & Vestergård Jørgensen, 1992). The EPC cell line is, however, very sensitive to several genotype IV isolates (United States Department of the Interior, 2007).

Detection of virus through the development of viral cytopathic effect (CPE) in cell culture is followed by virus identification through either antibody-based tests or nucleic acid-based tests. Any antibody-based tests would require the use of antibodies validated for their sensitivity and specificity (Ariel & Olesen, 2001). The use of the MAb IP5B11 (Lorenzen et al., 1988) is recommended as the reference reagent as all VHSV isolated to date react with this MAb.

##### 4.3.1.2.1.1. Virus extraction

In the laboratory the tissue in the tubes must be completely homogenised (either by stomacher, blender, validated homogeniser or most efficiently by mortar and pestle with sterile sand) and subsequently

suspended in the original transport medium. If a sample consists of whole fish less than 4 cm long, these should be minced with sterile scissors or scalpel after removal of the body behind the gut opening. If a sample consists of whole fish with a body length between 4 cm and 6 cm, the viscera, including the kidney, should be collected. If a sample consists of whole fish more than 6 cm long, tissue specimens should be collected as described in Section 3.4. The tissue specimens should be minced with sterile scissors or a scalpel and homogenised, as described above, and suspended in transport medium. The final ratio of tissue material to transport medium must be adjusted in the laboratory to 1:10.

The homogenate is centrifuged in a refrigerated centrifuge at 2–5°C at 2000–4000 *g* for 15 minutes and the supernatant is collected and treated for either 4 hours at 15°C or overnight at 4°C with antibiotics, e.g. gentamicin 1 mg ml<sup>-1</sup> may be useful at this stage. If the sample is shipped in transport medium (i.e. with exposure to antibiotics), treatment of the supernatant with antibiotics may be omitted. The antibiotic treatment aims at controlling bacterial contamination in the samples and makes filtration through membrane filters unnecessary.

If the collected supernatant is stored at –80°C within 48 hours of sampling it may be reused only once for virological examination.

Where practical difficulties arise (e.g. incubator breakdown, problems with cell cultures, etc.) that make it impossible to inoculate cells within 48 hours of collection of the tissue samples, it is acceptable to freeze the supernatant at –80°C and carry out virological examination within 14 days.

Prior to inoculating the cells, the supernatant is mixed with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of infectious pancreatic necrosis virus (IPNV) and the solution is incubated for a minimum of 1 hour at 15°C or a maximum of 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 (a virus that in some parts of Europe occurs in 50% of fish samples) aims at preventing 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 IPNV, treatment of inocula with antiserum to IPNV may be omitted.

#### 4.3.1.2.1.2. *Inoculation of cell monolayers*

BF-2 cells are grown at 20–24°C in suitable medium, e.g. Eagle's minimal essential medium (or modifications thereof) with a supplement of 10% fetal bovine serum 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.

Antibiotic-treated organ suspensions 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 (to prevent homologous interference). 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<sup>2</sup> 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 a similar or larger growth area are also acceptable.

#### 4.3.1.2.1.3. *Incubation of cell cultures*

Inoculated cell cultures are incubated at 15°C for 7–10 days. If the colour of the cell culture medium changes from red to yellow indicating medium acidification, pH adjustment with sterile bicarbonate solution or equivalent substances has to be performed to ensure cell susceptibility to virus infection.

At least every 6 months, or if decreased cell susceptibility is suspected, titration of frozen stocks of VHSV is performed to verify the susceptibility of the cell cultures to infection.

4.3.1.2.1.4. *Microscopy*

Inoculated cell cultures must be inspected regularly (at least three times a week) for the occurrence of CPE at  $\times 40$ – $150$  magnification. If obvious CPE is observed, virus identification procedures have to be initiated immediately. The use of a phase-contrast microscope is recommended.

4.3.1.2.1.5. *Subcultivation*

If no CPE has developed after the primary incubation for 7–10 days, subcultivation is performed with fresh cell cultures using a cell area similar to that of the primary culture.

Aliquots of medium (supernatant) from all cultures/wells constituting the primary culture are pooled according to the cell line 7–10 days after inoculation. The pools are then inoculated into homologous cell cultures undiluted and diluted 1/10 (resulting in final dilutions of 1/10 and 1/100, respectively, of the supernatant) as described above (Inoculation of cell monolayers).

Alternatively, aliquots of 10% of the medium constituting the primary culture are inoculated directly into a well with fresh cell culture (well-to-well subcultivation). In the case of salmonid samples, inoculation may be preceded by preincubation of the dilutions with an anti-IPNV antiserum at an appropriate dilution, as described above (Section 4.3.1.2.1.1 Virus extraction).

The inoculated cultures are then incubated for 7–10 days at  $15^{\circ}\text{C}$ , with observation, as described above (Section 4.3.1.2.1.4 Microscopy). If toxic CPE occurs within the first 3 days of incubation, subcultivation may be performed at that stage, but the cells must then be incubated for 7 days and subcultivated again with a further 7 days' incubation. When toxic CPE develops after 3 days, the cells may be passed once and incubated to achieve a total of 14 days from the primary inoculation. There should be no evidence of toxicity in the final 7 days of incubation.

If bacterial contamination occurs despite treatment with antibiotics, subcultivation must be preceded by centrifugation at  $2000$ – $4000\text{ g}$  for 15–30 minutes at  $2$ – $5^{\circ}\text{C}$ , and/or filtration of the supernatant through a  $0.45\text{ }\mu\text{m}$  filter (low protein-binding membrane). In addition to this, subcultivation procedures are the same as for toxic CPE.

If no CPE occurs, the test may be declared negative.

4.3.1.2.1.6. *Virus identification*

4.3.1.2.1.6.1. *Neutralisation test*

- i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge it at  $2000\text{ g}$  for 15 minutes at  $4^{\circ}\text{C}$ , or filter through a  $0.45\text{ }\mu\text{m}$  (or  $450\text{ nm}$ ) pore membrane to remove cell debris.
- ii) Dilute virus-containing medium from  $10^{-2}$  to  $10^{-4}$ .
- iii) Mix aliquots (for example  $200\text{ }\mu\text{l}$ ) 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  $15^{\circ}\text{C}$  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  $15^{\circ}\text{C}$ ; 24- or 12-well cell culture plates are suitable for this purpose, using a  $50\text{ }\mu\text{l}$  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 IFAT, an immunoperoxidase test, an ELISA or RT-PCR, 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 proven efficiency may be used alternatively.

#### 4.3.1.2.2. Antibody-based antigen detection methods

Antibody-based antigen detection methods, such as IFAT, ELISA and various immunohistochemical procedures for the detection of VHSV, have been developed over the years. It is generally accepted that the prime target organs are kidney, heart and spleen. These techniques can provide detection and identification relatively quickly compared with virus isolation in cell culture. However, various parameters, such as antibody sensitivity and specificity and sample preparation, can influence the results and a negative result should be viewed with caution. These techniques should not be used in attempts to detect carrier fish.

##### 4.3.1.2.2.1. Indirect fluorescent antibody test on imprints

- 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 the kidney pieces together with the other organs required for virus isolation in case this becomes necessary later.
- iv) Allow the imprint to air-dry for 20 minutes.
- v) Fix with acetone or ethanol/acetone and dry as indicated in Section 4.3.1.2.2.3, steps v–vii.
- vi) Rehydrate the above preparations (see Section 4.3.1.2.2.3, step ix) and block with 5% skim milk or 1% bovine serum albumin, in 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween-80 (PBST) for 30 minutes at 37°C.
- vii) Rinse four times with PBST.
- viii) Treat the imprints with the solution of antibody to VHSV and rinse as indicated in Section 4.3.1.2.2.3
- ix) Block and rinse as described previously in steps vi and vii.
- x) Reveal the reaction with suitable FITC-conjugated specific antibody, rinse and observe as indicated in Section 4.3.1.2.2.3, steps xii–xv.
- xi) If the test is negative, process the organ samples stored at 4°C for virus isolation in cell culture, as described above.

##### 4.3.1.2.2.2. Enzyme-linked immunosorbent assay (modified from Olesen & Jørgensen, 1991)

###### 4.3.1.2.2.2.1. ELISA procedure on tissue material

*Sampling procedures:* See Chapter 2.3.0 and the OIE *Handbook on Aquatic Animal Health Surveillance* (2009).

*Processing of organ samples:* See Chapter 2.3.0 and the OIE *Handbook on Aquatic Animal Health Surveillance* (2009).

###### 4.3.1.2.2.2.2. Follow procedure described below (ELISA procedure on cell culture supernatant)

- i) Set aside an aliquot of a 1/4 dilution of each homogenate in case further virus isolation in cell culture is required.
- ii) Treat the remaining part of the homogenate with 2% Triton X-100; mix gently.
- iii) Complete the other steps (v–xiii) of the procedure described in “ELISA procedure on cell culture supernatant”.
- iv) If the test is negative, process the organ samples stored at 4°C for virus isolation in cell culture, as described above.

4.3.1.2.2.3. *ELISA procedure on cell culture supernatant*

- i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of protein-A purified immunoglobulins (Ig) from rabbit anti sera against VHSV, in carbonate buffer, pH 9.6 (50  $\mu\text{l}$  well<sup>-1</sup>).
- ii) Incubate overnight at 4°C.
- iii) Rinse in PBS containing 0.05% Tween-20 (PBST).
- iv) Add 1% Triton X-100 to the virus suspension to be identified.
- v) Dispense 50  $\mu\text{l}$  well<sup>-1</sup> 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), 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 MAb to VHSV N protein (IP5B11) 50  $\mu\text{l}$  well<sup>-1</sup>.
- viii) Incubate for 1 hour at 37°C.
- ix) Rinse in PBST.
- x) Add to the wells (50  $\mu\text{l}$  well<sup>-1</sup>) 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 ortho-phenylene diamine and measure the absorbance at a wavelength of 492 nm.

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

4.3.1.2.2.3. *Indirect fluorescent antibody test*

- i) Prepare monolayers of cells in 2 cm<sup>2</sup> wells of cell culture plastic plates or on cover-slips in order to reach around 80% confluency, 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<sup>-1</sup> 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%/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<sup>2</sup> 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 2 cm<sup>2</sup> well<sup>-1</sup>.
- 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 proven efficiency may be used alternatively.

#### 4.3.1.2.3. Molecular techniques

Use of molecular tests (RT-PCR and real-time RT-PCR) is common because of their rapidity, sensitivity and specificity. In addition, with the presence of different strains with different pathogenic characteristics, discovery of the various North American, Asian, European and Atlantic marine strains, sequencing of PCR products can provide important epidemiological data (Einer-Jensen et al., 2002; Skall et al., 2005). Real-time RT-PCR tests are generally more sensitive than conventional RT-PCR tests. While use of these tests for virus detection and identification during the acute stage of disease has been justified for a number of years, their sensitivity and specificity compared with virus isolation by cell culture and subsequent identification have only recently been definitively established for a real-time RT-PCR assay (Garver et al., 2011; Jonstrup et al., 2013). These two assays have been found to be suitable for identification of VHSV of all genotypes.

At the time of writing (2011), loop-mediated isothermal amplification (LAMP) for VHSV cannot be recommended as a general assay for detection of VHSV as the only published LAMP is not validated against all genotypes, and by aligning sequences it is likely that the published LAMP will not recognise all genotypes (Soliman & El-Matbouli, 2006).

##### 4.3.1.2.3.1. Preparation of viral RNA

All work with RNA should be performed on ice, using gloves.

Collect aliquots of culture medium from infected monolayer cells exhibiting CPE. Centrifuge at 1000 *g* for 5 minutes to remove cell debris.

RNA is extracted using the phenol-chloroform method or by RNA affinity spin columns, according to the manufacturer's instructions. RNA must be resuspended in distilled RNase-free water (e.g. water treated with 0.1% diethyl pyrocarbonate).

##### 4.3.1.2.3.2. Conventional reverse-transcription polymerase chain reaction (RT-PCR)

The RT-PCR amplification can be performed in one or two steps. Both procedures are based on a PCR amplification step using primers: 5'-ATG-GAA-GGA-GGA-ATT-CGT-GAA-GCG-3' (VN forward) and 5'-GCG-GTG-AAG-TGC-TGC-AGT-TCC-C-3' (VN reverse) (Snow et al., 2004). VN Forward and VN reverse are designated to amplify a region corresponding to bases 1–505 of the VHSV N-gene (Snow et al., 2004).

**NOTE:** Genotype IVb isolates may not be recognised using these primers; the use of real-time RT-PCR or antibody-based methods is therefore recommended for confirmation.

###### 4.3.1.2.3.2.1. One-step RT-PCR

A 50 µl single tube RT-PCR can be performed using, e.g. Qiagen OneStep RT-PCR System (Qiagen, Germany), according to the manufacturer's instructions. Briefly, the reaction mixture consists of: 5 µl of extracted viral RNA, 2 µl 10 mM dNTP, 10 µl 5 × RT-PCR reaction buffer (with 12.5 mM MgCl<sub>2</sub>) and 2 µl enzyme mix. The RT-PCR can be performed using VN Forward and VN reverse primers at a final concentration of 0.6 µM each. The following cycles are recommended: 50°C for 30 minutes, 95°C for 15 minutes, 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 60 seconds. Subsequently, the reaction is held at 68°C for 7 minutes.

###### 4.3.1.2.3.2.2. Two-step RT-PCR

A 20 µl cDNA synthesis can be performed using, for example, iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Briefly, the reaction mixture consists of: 5 µl of extracted viral RNA, 4 µl of 5× iScript Reaction Mix, 1 µl iScript Reverse Transcriptase, and 10 µl Nuclease-free water. Incubate samples using the following programme: 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes. Use 5 µl for PCR.

A 25 µl PCR can be performed after cDNA synthesis (described above) using, for example, Go Taq Flexi Polymerase (Promega), according to manufacturer's instructions. Briefly, the reaction mixture consists of: 5 µl of cDNA, 11.125 µl Milli Q water, 5 µl 5× Green GoTaq Flexi buffer, 2.5 µl 25 mM MgCl<sub>2</sub>, 0.5 µl VN Forward (10 µM), 0.5 µl VN Reverse (10 µM), 0.25 µl 25 mM (of each) dNTP, 0.125 µl Go Taq Flexi DNA polymerase. Incubate samples using the following programme: 94°C for 5 minutes, 36 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 60 seconds. Subsequently, the reaction is held at 68°C for 7 minutes.

Quantity and specificity of the one-step and the two-step RT-PCR reactions can be evaluated by gel electrophoresis of a 1/10 reaction in 1.5% agarose gel with ethidium bromide and observed using UV transillumination.

**NOTE:** The PCR can vary depending on the conditions under which it is performed, e.g. the thermal cycling protocols might need optimisation, depending on the thermal cycler in use. Furthermore, false-positive results can occur because of, for example, false primer annealing or laboratory contamination. Care should also be taken when the virus is grown on BF-2 cells, since on rare occasions a false positive band with a size slightly smaller than a true VHSV band can be observed. It is therefore important to include adequate positive and negative controls and amplicons must be sequenced should there be any doubts.

#### 4.3.1.2.3.3. Real-time RT-PCR

The RT-PCR amplification can be performed using primers/probe adapted from Jonstrup et al., 2013: 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 probe: 5'-FAM-TAG-AGG-GCC-TTG-GTG-ATC-TTC-TG-BHQ1. These primers target nt. 532–608 according to GenBank accession number Z93412. Alternatively primers/probe can be adapted from Garver et al., 2011: Forward primer (2F): 5'-ATG-AGG-CAG-GTG-TCG-GAG-G-3'; Reverse primer (2R): 5'-TGT-AGT-AGG-ACT-CTC-CCA-GCA-TCC-3'; and probe (2-MGB): 5'-FAM-TAC-GCC-ATC-ATG-ATG-AGT-MGBNFQ-3' targeting nt. 787-868 according to GenBank accession number Z93412.

##### 4.3.1.2.3.3.1. One-step real-time RT-PCR

A 25 µl single tube RT-PCR can be performed using, e.g. Quantitect Probe RT-PCR kit (Qiagen, Germany), according to the manufacturer's instructions. Briefly, the reaction mixture consists of: 5 µl of extracted viral RNA (approximately 0.05–2 µg), 12.5 µl 2×QRT-PCR master mix, and 0.25 µl RT/RNase block enzyme mix. The real-time RT-PCR can be performed using forward and reverse primers at a final concentration of 0.9 µM each, and probe at a final concentration of 0.25 µM. PCR program depends on the kit and real-time PCR equipment used. For a mx3005p (Stratagene) using Quantitect Probe RT-PCR kit (Qiagen, Germany) run following program: 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, if using a different platform and/or kit adjust if necessary.

Please note that the sensitivity of the real-time RT-PCR assay depends very much on the kit used (see Jonstrup et al., 2013 for further details).

## 4.3.2. Serological methods

Surveillance based on serological tests has several advantages compared with virus isolation, especially in cases where water temperature is too high for virus isolation and in endemically infected populations without clinical signs of disease. The antibody response can, however, first be detected 3–4 weeks after infection. On the other hand, high antibody levels will persist for a long time: > 6 months after infection (Fregeneda-Grandes & Olesen, 2007; Lapatra, 1996; Lorenzen & Lapatra, 1999). The disadvantage of serological tests is the slow development of fish antibodies following infection, especially at low water temperatures. A final assessment of test sensitivity, specificity and reproducibility of serological methods for detection in fish of antibodies against VHSV is being undertaken. When properly assessed and validated the methods will be inserted in the present chapter.

## 5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of VHSV are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These recommendations are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

**Table 5.1. Methods for surveillance, detection and diagnosis**

Method	Targeted surveillance			Presumptive diagnosis	Confirmatory diagnosis
	Larvae	Juveniles	Adults		
Gross signs	d	d	d	b	d
Histopathology	d	d	d	b	d
Transmission EM	d	d	d	c	d
Isolation in cell culture followed by one of the identification methods	a	a	a	a	a
Antibody-based assays	d	d	d	b	a
Conventional RT-PCR followed by sequencing	c	c	c	b	a
Real-time RT-PCR	c	c	c	a	a

EM = electron microscopy; RT-PCR = reverse transcriptase polymerase chain reaction.

## 6. Tests recommended for targeted surveillance to declare freedom from VHS

The test recommended for targeted surveillance is cultivation of fish tissue specimens on BF-2 cells with subsequent virus identification by immunochemical or nucleic-acid-based tests as described in Section 4 of this chapter.

## 7. Corroborative diagnostic criteria

### 7.1. Definition of suspect case

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

- i) The presence of post-mortem findings consistent with VHS, with or without clinical signs of disease. Post-mortem findings and clinical signs of disease shall be in accordance with those described in Sections 4.1 and 4.2 of this chapter;
- ii) VHSV-typical CPE in cell cultures before confirmation;
- iii) Where an investigation reveals epidemiological links to VHS suspected or confirmed farms: detection of antibodies to VHSV in fish.

Suspicion of VHS can be ruled out if continued investigations reveal no further evidence of the presence of the pathogen.

## 7.2. Definition of confirmed case

The presence of VHSV is considered to be confirmed if, in addition to the criteria in Section 7.1, one or more of the following criteria are met:

- i) VHSV isolation is carried out in cell culture followed by virus identification by either an antibody-based test (IFAT, ELISA, neutralisation test, immunohistochemistry) and/or RT-PCR followed by the sequencing of the amplicon or by real-time RT-PCR;
- ii) VHSV is detected in tissues or tissue preparations by immunoassay using specific anti-VHSV antibodies;
- iii) Detection of VHSV in tissue preparations by RT-PCR followed by sequencing of the amplicon or by real-time RT-PCR.

The confirmation of the first case of VHS in farms in non-infected zones or compartments must not be based on ii) or iii) alone.

The tissue material for virological examination may, in some cases, be accompanied by supplementary material for bacteriological, parasitological, histological or other examination to allow for a differential diagnosis.

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**NB:** There are OIE Reference Laboratories for Viral haemorrhagic septicaemia  
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:  
<http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).  
Please contact the OIE Reference Laboratories for any further information on Viral haemorrhagic septicaemia