# 2.2.7 Viral Hemorrhagic Septicemia

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# A. Name of Disease and Etiological Agent

Viral hemorrhagic septicemia (VHS) is one of the most important viral diseases of finfish worldwide. In the past, VHS was thought to affect mainly Rainbow Trout, *Oncorhynchus mykiss*, reared at freshwater facilities in Western Europe where it was known by various names including Egtved disease and infectious kidney swelling and liver degeneration (Wolf 1988). Today, VHS is known as a cause of mortality in cultured and wild fish in freshwater and marine environments in several regions of the northern hemisphere (Meyers and Winton 1995; Marty et al. 1998; Dixon 1999; Smail 1999; Takano et al. 2001; Marty et al. 2003; Skall et al. 2005b; Elsayed et al. 2006; Gagné et al. 2007; Lumsden et al. 2007; Kim and Faisal 2011). Viral hemorrhagic septicemia is caused by infection with viral hemorrhagic septicemia virus (VHSV); VHSV is assigned to the order *Mononegavirales*, family *Rhabdoviridae*, genus *Novirhabdovirus* and species *Piscine novirhabdovirus* (Walker et al. 2018). The VHSV genome is comprised of 11,158 bases and six genes, including nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonstructural viral protein (Nv) and polymerase (L), arranged in the order 3' – N – P – M – G – Nv – L – 5' (Schütze et al. 1999).

# B. Known Geographical Range and Host Species of the Disease

## 1. Geographical Range

Viral hemorrhagic septicemia virus is endemic among marine and freshwater fish in temperate climates of the northern hemisphere, including Western Europe, North America and Eastern Asia (Escobar et al. 2018). Countries or regions where VHSV has been isolated from natural or experimental infections of fish using cell culture methods and confirmed by serological or molecular assays are listed in Table 1.

Nucleotide sequence analysis of the N- and G-genes has revealed the presence of four main genotypes (I – IV) (Snow et al. 1999; Einer-Jensen et al. 2004) (Table 1). Genotypes I – III occur predominantly within Europe and genotype IV occurs in North America, South Korea, Japan, and Iceland. The order of genotypes reflects their discovery and naming and does not imply the sequential spread of the virus. Numerous hypotheses have been raised on the sequential spread of the virus through the northern hemisphere (Einer-Jensen et al. 2004; Studer and Janies 2011; Pierce and Stepien 2012; He et al. 2014).

Genotype I: This genotype is best known for causing mortality in freshwater-reared Rainbow Trout in Europe. Molecular genetic data suggest that this genotype originated from wild marine fish in Europe with several host species jumps before adaptation to Rainbow Trout (Einer-Jensen et al. 2004). This genotype is divided into six sublineages including Ia-1, Ia-2, Ib, Ic, Id & Ie, and the sublineages correspond with sequence differences in the virus from fish in specific geographic regions within Europe (Kahns et al. 2012). Genotype I infections occur in freshwater trout, Northern Pike, *Esox lucius*, and Largemouth Bass, *Micropterus salmoides*, and in a variety of wild marine fish from Europe. A single Ib isolate was found in Japan and thought to have been a foreign introduction (Nishizawa et al. 2002).

Genotype II: Isolated from wild marine fish from the Baltic Sea (Einer-Jensen et al. 2004).

Genotype III: Isolated from wild marine fish from Scottish waters, the Skagerrak Strait near Norway, and from Greenland halibut, *Reinhardtius hippoglossoides*, caught at the Flemish Cap, a fishing ground in the North Atlantic Ocean near Newfoundland. This genotype is also associated with mortality in Turbot farms in the British Isles and marine-raised Rainbow Trout in Norway and Finland (Snow et al. 1999; Einer-Jensen et al. 2004; Lopez-Vazquez et al. 2006; Raja-Halli et al. 2006; Dale

et al. 2009; Ito et al. 2016). In the Shetland Islands in Scotland, this genotype caused mortality in various wrasse spp. used as cleaner fish in a fish farm (Munro et al. 2015).

Genotype IV: Isolated mainly from wild marine fish from the Pacific northwest coastal area of North America with occasional spill over into domestic production, wild marine fish from the Atlantic coastal area, and from freshwater fish from the Great Lakes region of North America. Four sublineages occur. Genotype IVa was originally found in wild marine fish in the Pacific northwestern coast of North America, in Asia (Nishizawa et al. 2002), in Atlantic herring in 2003 (Elsayed et al. 2006) and most recently in Atlantic Canada (CFIA 2016). In the Pacific Northwest, Pacific Herring, *Clupea pallasii*, are a common and highly susceptible host species (Hershberger et al. 2016), with occasional spill over to net-pen farmed Atlantic Salmon, *Salmo salar* (Traxler et al. 1995). Atlantic Salmon may show similar clinical signs as other susceptible species, though are less severely affected and often show little to no signs of disease (Lovy et al. 2013). In Japan and Korea, genotype IVa occurs in wild and farmed Japanese Flounder, *Paralichthys olivaceus* (Kim et al. 2009). Sublineage IVb occurs in freshwater fish from the Great Lakes (Elsayed et al. 2006). Two other sublineages occur in the North Atlantic Ocean, including IVc in estuarine fish from Atlantic Canada (Gagné et al. 2007), and IVd from wild and farmed Lumpfish, *Cyclopterus lumpus*, from Iceland (Guðmundsdóttir et al. 2019).

**2. Host Species**

Over 100 species of freshwater and marine fish have been reported to be naturally or experimentally susceptible to VHSV (Tables 1 and 2). Various species of freshwater turtles have been shown to harbor the virus in internal organs up to 20 days after feeding on fish experimentally infected with VHSV, suggesting that turtles may be vectors of the virus (Goodwin and Merry 2011a). In addition, invertebrates including leech *Myzobdella lugubris*, amphipod *Diporeia* spp., and cladoceran *Moina macrocopa* have been found to harbor the virus (Faisal and Schulz 2009; Faisal and Winters 2011; Ito and Olesen 2017), but their role in pathogen transmission has not been established. While large outbreaks of disease associated with high mortality have occurred in aquaculture facilities and in some populations of wild fish, VHSV has also been isolated from fish that appeared normal.

## C. Epizootiology

VHSV is largely believed to be a virus of marine origin. All genotypes have been isolated from marine fish, whereas to date only genotype I in Europe and IVb from the Great Lakes occurs in fish in a freshwater environment. The virus is more stable in freshwater compared to seawater, remaining infective for up to 13 days in freshwater compared to only 4 days in seawater at 15°C (Hawley and Garver 2008). VHSV is readily transmissible to susceptible fish of all ages. The main portal of entry is believed to be the epithelial tissues of the gills or skin, especially at the base of the fins (Harmache et al. 2006). Disease outbreaks are typically seen at water temperatures from 9-12°C, while chronic fish losses resulting in large-scale mortality may occur at lower temperatures up to 5°C. As temperature increases, mortality and the proportion of virus carriers decreases. At temperatures above 15°C, mortality from VHS is typically low (Goodwin and Merry 2011b). Infected fish mount a strong antibody response in survivors of epizootics (Millard and Faisal 2012a; 2012b; Faisal et al. 2019). A neurologic form of disease may develop (Ghittino 1965). Persistence of VHS genotype I has been demonstrated in brain of Rainbow Trout over 1 year after infection (Neukirch 1986) and in genotype IVa for at least 224 days in Pacific Herring following infection (Hershberger et al. 2010). Further, in a natural epizootic of VHS genotype IVa in laboratory-held Pacific Herring, chronic neurological disease manifested following an episode of acute disease (Lovy et al. 2012).

**Table 1. Natural Host*, Genotype, and Geographic Range of Viral Hemorrhagic Septicemia Virus.**

| Genotype | Common name | Scientific name | Geographic location | Reference |
|---|---|---|---|---|
| Ia<br>IVa | Atlantic Salmon | *Salmo salar* | Spain;<br>Pacific Coast - North America | Jimenez de la Fuente et al. 1988; Traxler et al. 1995 |
| Ia<br>Ib<br>IVc | Brown Trout | *Salmo trutta* | Western Europe;<br>Atlantic Coast - North America | de Kinkelin & Le Berre 1977; Thiery et al. 2002; Gagné et al. 2005 |
| Ia | Grayling | *Thymallus thymallus* | Switzerland;<br>Italy | Meier & Wahli 1988; Cieslak et al. 2016 |
| Ia, IVb | Lake Trout | *Salvelinus namaycush* | Immersion challenge;<br>Inland Lakes - North America | Dorson et al. 1991; Thompson et al. 2011 |
| Ia<br>IVb | Largemouth Bass | *Micropterus salmoides* | France;<br>Great Lakes - North America | de Kinkelin et al. 1999; Thiery et al 2002; Faisal et al. 2012 |
| Ia | Marble Trout | *Salmo marmoratus* | Slovenia | Pascoli et al. 2015 |
| Ia<br>IVb | Northern Pike | *Esox lucius* | Western Europe;<br>Great Lakes - North America | Meier & Jorgensen 1979; Thompson et al. 2011 |
| Ia-Ie<br>II<br>III<br>IVb | Rainbow Trout | *Oncorhynchus mykiss* | Europe (most countries);<br>Northeastern US | Wolf 1988; Skall et al. 2005b; Dale et al 2009; Thompson et al. 2011; Schönherz et al. 2018 |
| Ia | Whitefish | *Coregonus lavaretus* | Switzerland;<br>Germany | Meier et al. 1994<br>Cieslak et al. 2016 |
| Ib<br>III | Atlantic Cod | *Gadus morhua* | Baltic Sea; North Sea;<br>North Atlantic | Jensen et al. 1979; Mortensen et al. 1999; Smail 2000; King et al. 2001 |
| Ib<br>II<br>III<br>IVa | Atlantic Herring | *Clupea harengus* | Baltic Sea; English Channel; Kattegat; Skagerrak, North Sea; Japan; Maine; Atlantic Canada | Dixon et al. 1997; Mortensen et al. 1999; King et al. 2001; Nishizawa et al. 2002; Elsayed et al. 2006; CFIA 2016 |
| Ib<br>III | Blue Whiting | *Micromesistius poutassou* | North Sea | Mortensen et al. 1999; Cieslak et al. 2016 |
| Ib | Common Dab | *Limanda limanda* | Kattegat; Baltic Sea | Skall et al. 2005a |
| Ib | European Flounder | *Platichthys flesus* | Baltic Sea | Skall et al. 2005a |
| Ib | European Plaice | *Pleuronectes platessa* | Skagerrak; Kattegat | Skall et al. 2005a |
| Ib<br>II | European Sprat | *Sprattus sprattus* | Baltic Sea | Mortensen et al. 1999; Schönherz et al. 2018 |
| Ib<br>IVa | Japanese (Olive) Flounder | *Paralichthys olivaceus* | Japan; Korea | Takano et al. 2000, 2001; Nishizawa et al. 2002; Kim et al. 2009 |
| Ib | Sand Goby | *Pomatoschistus minutus* | Baltic Sea | Skall et al. 2005a |
| Ib<br>Ie<br>III | Turbot | *Scophthalmus maximus*<br>*Psetta maxima* | Germany; Gigha (Scotland);<br>Ireland;<br>Black Sea (Turkey) | Schlotfeldt et al. 1991; Ross et al. 1994; Nishizawa et al. 2006; Schönherz et al. 2018 |

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|---|---|---|---|---|
| Ie | Anchovy | *Engraulis encrasicolus* | Black Sea | Ogut & Altuntas 2014a |
| Ie | Mediterranean Horse Mackerel | *Trachurus mediterraneus* | Black Sea | Ogut & Altuntas 2014a |
| Ie | Pilchard | *Sardina pilchardus* | Black Sea | Ogut & Altuntas 2014a |
| Ie | Pontic Shad | *Alosa immaculata* | Black Sea | Ogut & Altuntas 2014a |
| Ie | Red Mullet | *Mullus barbatus* | Black Sea | Ogut & Altuntas 2014a |
| Ie, IVb | Round Goby | *Neogobius melanostomus* | Great Lakes - North America; Black Sea | Groocock et al. 2007; Ogut & Altuntas 2014a |
| Ie | Stargazer | *Uranoscopus scaber* | Black Sea | Ogut & Altuntas 2014a |
| Ie | Thornback Ray | *Raja clavata* | Black Sea | Ogut & Altuntas 2014a |
| Ie | Three-bearded Rockling | *Gaidropsarus vulgaris* | Black Sea | Ogut & Altuntas 2014a |
| Ie<br>III | Whiting | *Merlangius merlangus* | Black Sea<br>North Sea | Ogut & Altuntas 2014a<br>Mortensen et al. 1999; King et al. 2001 |
| II | River Lamprey | *Lampetra fluviatilis* | Finland | Gadd et al. 2010; Schönherz et al. 2018 |
| III | Ballan Wrasse | *Labrus bergylta* | Aquaculture facility | Munro et al. 2015 |
| III | Corkwing Wrasse | *Symphodus melops* | Aquaculture facility | Munro et al. 2015 |
| III | Cuckoo Wrasse | *Labrus mixtus* | Aquaculture facility | Munro et al. 2015 |
| III | Goldsinny Wrasse | *Ctenolabrus rupestris* | Aquaculture facility | Munro et al. 2015 |
| III | Norway Pout | *Trisopterus esmarkii* | North Sea; North Atlantic; Skagerrak | Mortensen et al. 1999; King et al. 2001 |
| III | Rock Cook Wrasse | *Centrolabrus exoletus* | Aquaculture facility | Munro et al. 2015 |
| III | Senegalese Sole | *Solea senegalensis* | Experimental | Vazquez et al 2016 |
| IVa<br>IVb | Chinook Salmon | *Oncorhynchus tshawytscha* | Pacific Coast;<br>Great Lakes - North America | Winton et al. 1991;<br>Faisal et al. 2012 |
| IVa | Coho Salmon | *Oncorhynchus kisutch* | Pacific Coast - North America | Winton et al. 1991 |
| IVa | Eulachon | *Thaleichthys pacificus* | Pacific Coast - North America | Hedrick et al. 2003 |
| IVa | Pacific Cod | *Gadus macrocephalus* | Pacific Coast - North America | Meyers et al. 1992 |
| IVa | Pacific Herring | *Clupea pallasi* | Pacific Coast - North America | Meyers et al. 1994; Kocan et al. 2001 |
| IVa | Pacific Mackerel | *Scomber japonicus* | Pacific Coast - North America | Hedrick et al. 2003 |
| IVa | Pacific Sand Lance | *Ammodytes hexapterus* | Pacific Coast - North America | Kocan et al. 2001 |
| IVa | Pacific Sardine (Pilchard) | *Sardinops sagax* | Pacific Coast - North America | Hedrick et al. 2003 |
| IVa | Shiner Perch | *Cymatogaster aggregata* | Pacific Coast - North America | Hedrick et al. 2003 |
| IVa | Steelhead | *Oncorhynchus mykiss* | Pacific Coast - North America | Winton et al. 1991 |
| IVa | Zebrafish | *Danio rerio* | Immersion challenge | Novoa et al. 2006; Cuong & Thoa. 2020 |
| IVb | Black Crappie | *Pomoxis nigromaculatus* | Great Lakes - North America | Faisal et al. 2012 |
| IVb | Bluntnose Minnow | *Pimephales notatus* | Great Lakes - North America | USDA-APHIS 2008 |
| IVb | Bluegill | *Lepomis macrochirus* | Great Lakes - North America | Faisal et al. 2012 |
| IVb | Brown Bullhead | *Ameiurus nebulosis* | Great Lakes - North America | Thompson et al. 2011 |
| IVb | Emerald Shiner | *Notropis atherinoides* | Great Lakes - North America | Thompson et al. 2011; Boonthai et al. 2018 |
| IVb | Fathead Minnow | *Pimephales promelas* | Great Lakes - North America | Al-Hussinee et al. 2010 |
| IVb | Freshwater Drum | *Aplodinotus grunniens* | Great Lakes - North America | Lumsden et al. 2007; Faisal et al. 2012 |
| IVb | Gizzard Shad | *Dorosoma cepedianum* | Great Lakes - North America | Thompson et al. 2011 |
| IVb | Lake Cisco | *Coregonus artedi* | Great Lakes - North America | Thompson et al. 2011; Weeks et al. 2011 |
| IVb | Lake Whitefish | *Coregonus clupeaformis* | Great Lakes - North America | Faisal et al. 2012 |
| IVb | Muskellunge | *Esox masquinongy* | Great Lakes - North America | Elsayed et al. 2006 |

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| IVb | Pumpkinseed | *Lepomis gibbosus* | Great Lakes - North America | Faisal et al. 2012 |
| IVb | Rock Bass | *Ambloplites rupestris* | Great Lakes - North America | Faisal et al. 2012 |
| IVb | Smallmouth Bass | *Micropterus dolomieu* | Great Lakes - North America | Faisal et al. 2012 |
| IVb | Spottail Shiner | *Notropis hudsonius* | Great Lakes - North America | Kim & Faisal 2011 |
| IVb | Walleye | *Sander vitreus* | Great Lakes - North America | Thompson et al. 2011 |
| IVb | White Bass | *Morone chrysops* | Great Lakes - North America | Thompson et al. 2011 |
| IVb | White Perch | *Morone americana* | Great Lakes - North America | Thompson et al. 2011 |
| IVb | Yellow Perch | *Perca flavescens* | Great Lakes - North America | Kane-Sutton et al. 2009 |
| IVc | Mummichog | *Fundulus heteroclitus* | Atlantic Coast - North America | Olivier 2002 |
| IVc | Striped Bass | *Morone saxatilis* | Atlantic Coast - North America | Gagne et al. 2007 |
| IVc | Threespine Stickleback | *Gasterosteus aculeatus* | Pacific Coast & Atlantic Coast - North America | Kent et al. 1998; Olivier 2002 |
| IVd | Lumpfish | *Cyclopterus lumpus* | Iceland | Guðmundsdóttir et al. 2019 |

*Host species listed as susceptible are based on OIE criteria (link below).
<https://www.oie.int/index.php?id=171&L=0&htmfile=chapitre_criteria_species.htm>

**Table 2. Host species* with incomplete evidence for susceptibility, Genotype, and Geographic Range of Viral Hemorrhagic Septicemia Virus.**

| Genotype | Common name | Scientific name | Geographic location | Reference |
|---|---|---|---|---|
| Ia | Arctic Char | *Salvelinus alpinus* | Immersion challenge | Dorson et al. 1991 |
| Ia | Rainbow Trout hybrids | *Oncorhynchus mykiss* | Immersion challenge | Dorson et al. 1991 |
| Ib | Four-bearded Rockling | *Rhinonemus cimbrius* | Baltic Sea | Mortensen et al. 1999; Cieslak et al. 2016 |
| Ib | Japanese Sand Lance | *Ammodytes personatus* | Japan | Watanabe et al. 2002 |
| Ib | Mebaru (Black Rockfish) | *Sebastes inermis* | Japan | Isshiki et al. 2003 |
| Ib | Oblong Rockfish | *Sebastes oblongus* | Japan | Isshiki et al. 2010 |
| Ib | Rock Gunnel | *Pholis gunnellus* | Sweden | Schönherz et al. 2018 |
| Ib | Silvery Pout | *Gadiculus argenteus* | Norway | Sandlund et al. 2014 |
| Ie | Black Scorpionfish | *Scorpaena porcus* | Black Sea | Ogut & Altuntas 2014a |
| Ie | Brook Trout | *Salvelinus fontinalis* | Poland | Ogut & Altuntas 2011 |
| Ie | European Seabass | *Dicentrarchus labrax* | Black Sea | Ogut &Altuntas2014b |
| Ie | Garfish | *Belone belone* | Black Sea | Ogut &Altuntas 2014a |
| III | Atlantic Halibut | *Hippoglossus hippoglossus* | Immersion, cohabitation | Bowden 2003 |
| III | European Eel | *Anguilla anguilla* | France; Germany | Castric et al. 1992; Cieslak et al. 2016 |
| III | Gray Gunnard | *Eutrigla gurnardus* | North Sea | Wallace et al. 2015 |
| III | Greenland Halibut | *Reinhardtius hippoglossoides* | Flemish Cap - North Atlantic | Dopazo et al. 2002 |
| III | Haddock | *Melanogrammus aeglefinus* | North Sea | Smail 2000 |
| III | Lesser Argentine | *Argentina sphyraena* | North Sea | Mortensen et al. 1999 |
| III | Poor Cod | *Trisopterus minutus* | North Atlantic | King et al. 2001 |
| IVa | Amoured Cusk | *Hoplobrotula armata* | South Korea | Lee et al. 2007 |
| IVa | Claudy Catshark | *Scyliorhinus torazame* | South Korea | Lee et al. 2007 |
| IVa | Cubed Snailfish | *Liparis tessellatus* | South Korea | Lee et al. 2007 |
| IVa | English Sole | *Parophrys vetula* | Pacific Coast - North America | Hershberger et al. 1999 |

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| IVa | Flathead Grey Mullet | *Mugil cephalus* | South Korea | Lee et al. 2007 |
| IVa | Izu Scorpianfish | *Scorpaena izensis* | South Korea | Lee et al. 2007 |
| IVa | Largehead Hairtail | *Trichiums lepturus* | South Korea | Lee et al. 2007 |
| IVa | Marine Medaka | *Oryzias dancena* | South Korea | Kim et al. 2013 |
| IVa | Pacific Hake | *Merluccius productus* | Pacific Coast - North America | Meyers et al. 1999 |
| IVa | Pacific Tomcod | *Microgadus proximus* | Pacific Coast - North America | Meyers et al. 1999 |
| IVa | Sablefish (Black Cod) | *Anoplopoma fimbria* | Pacific Coast - North America | Hedrick et al. 2003 |
| IVa | Silver Pomfret | *Pampus argenteus* | South Korea | Lee et al. 2007 |
| IVa | Surf Smelt | *Hypomesus pretiosus* | Pacific Coast - North America | Hedrick et al. 2003 |
| IVa | Tube-Snout | *Aulorhynchus flavidus* | Pacific Coast - North America | Traxler et al. 1995 |
| IVa | Walleye Pollock | *Theragra chalcogramma* | Pacific Coast - North America | Meyers et al. 1999 |
| IVa | Yellow Croaker | *Larimichthys polyactis* | South Korea | Lee et al. 2007 |
| IVb | Banded Killifish | *Fundulus diaphanus* | Great Lakes - North America | Bain et al. 2010 |
| IVb | Burbot | *Lota lota* | Great Lakes - North America | Thompson et al. 2011 |
| IVb | Channel Catfish | *Ictalurus punctatus* | Great Lakes - North America | Thompson et al. 2011 |
| IVb | Common Carp | *Cyprinus carpio* | Inland Lakes - North America | Thompson et al. 2011 |
| IVb | Golden Shiner | *Notemigonus crysoleucas* | Immersion challenge | Cornwell et al. 2013 |
| IVb | Japanese Fluvial Sculpin | *Cottus pollux* | Japan | Ito & Olesen 2013 |
| IVb | Japanese Rice Fish | *Oryzias latipes* | Japan | Ito & Olesen 2013 |
| IVb | Sea Lamprey | *Petromyzon marinus* | Great Lakes - North America | Thompson et al. 2011 |
| IVb | Shorthead Redhorse Sucker | *Moxostoma macrolepidotum* | Great Lakes - North America | Faisal et al. 2012 |
| IVb | Silver Redhorse Sucker | *Moxostoma anisurum* | Great Lakes - North America | Faisal et al. 2012 |
| IVb | Tiger Muskellunge | *Esox lucius* $X_{SEP}$ *E. masquinongy* hybrids | Feeding infected minnows | Getchell et al. 2013 |
| IVb | Trout-perch | *Percopsis omiscomaycus* | Great Lakes - North America | Thompson et al. 2011 |
| IVb | White Crappie | *Pomoxis annularis* | Great Lakes - North America | Al-Hussinee et al. 2011 |
| IVb | Yoshinobori | *Rhinogobius sp.* | Japan | Ito & Olesen 2013 |
| IVb | Piscicolid leech | *Myzobdella lugubris* | Great Lakes - North America | Faisal & Schulz 2009 |
| IVb | Amphipods | *Diporeia* sp. | Great Lakes - North America | Faisal & Winters 2012 |

*These host species have incomplete evidence for susceptibility. If there is incomplete evidence to demonstrate susceptibility of a species, the *Competent Authority* should, prior to the implementation of any import health measures for the species, undertake a *risk assessment* for the *pathogenic agent* under consideration, in accordance with the recommendations in OIE Chapter 2.1.

## D. Disease Signs

### 1. Behavioral Signs Associated with the Disease

A variety of clinical signs may be apparent in infected fish (Wolf 1988; Smail 1999; Brudeseth et al. 2005; Lumsden et al. 2007; Kim and Faisal 2010). Some fish can show profound clinical manifestation whereas others appear to be nearly normal. Fish may be lethargic or hyperactive during acute disease. Neurologic behavioral signs, more common in chronic disease, may include corkscrew or irregular swimming behavior.

### 2. External Gross Signs

External clinical signs of disease include hemorrhage in the skin, base of the fins, eyes and gills (Figure 1), exophthalmia, abdominal distention, darkened coloration and anemia. Severely pale gills as a result of anemia is often seen in freshwater fish from the Great Lakes infected with genotype IVb (Figure 2). In chronic or neurologic manifestation, most of these disease signs may be absent while dark dorsal coloration may be the only external gross sign.


**Figure 1.** External disease signs of VHSV genotype IVa in Pacific Herring. Gross signs include (A) hemorrhage at the base of the fins, (B) hyperemia, particularly around the head, and (C) skin hemorrhages.

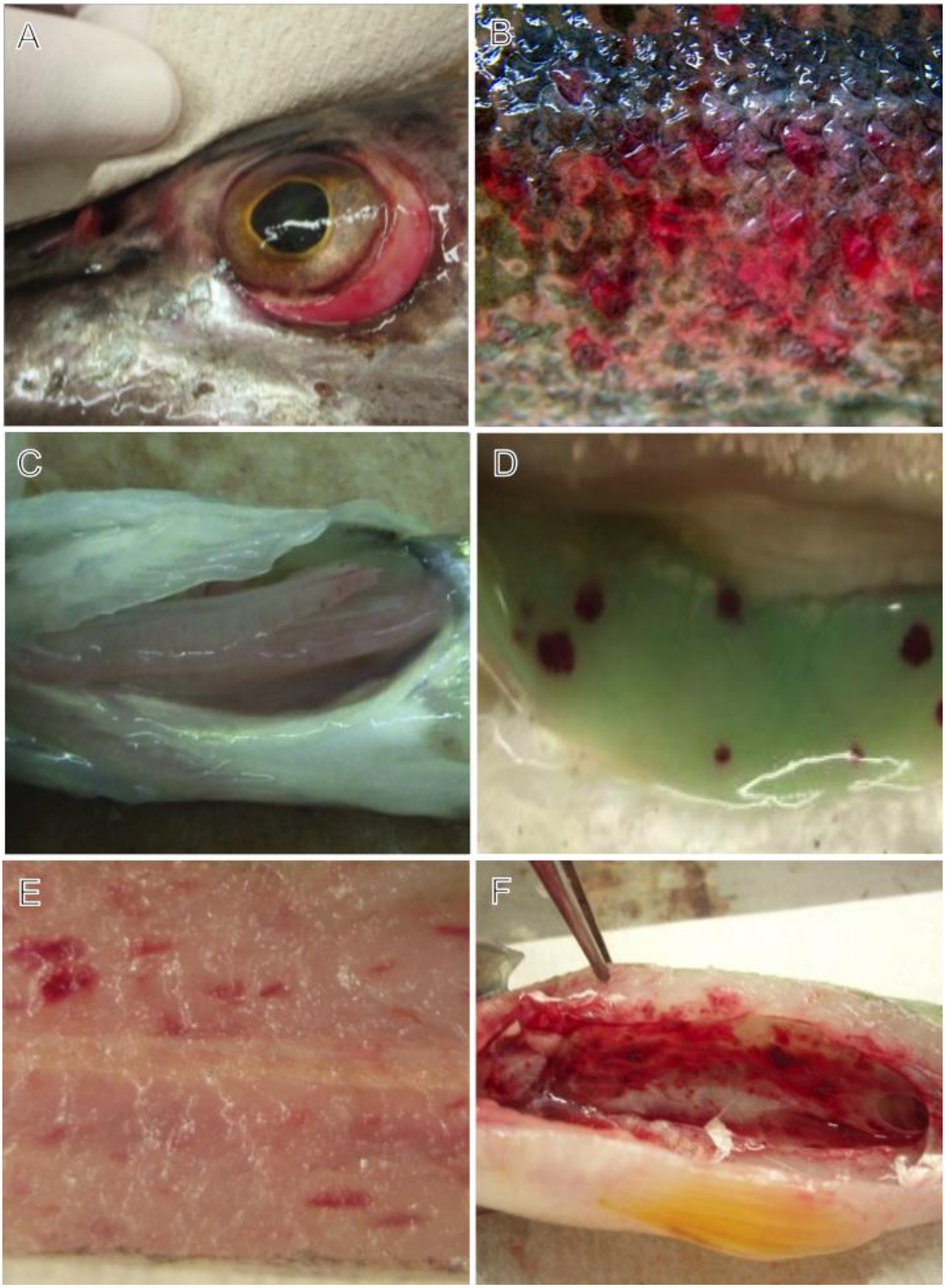


**Figure 2.** Gross lesions related to VHSV IVb in Muskellunge (A,B,C,D), Freshwater Drum (E), and Lake Herring (F), including periorbital erythema (A), skin hemorrhages (B), pale gills (C), liver (D), multifocal hemorrhages in the skeletal muscle (E), and the inner wall of the swimbladder (F).

**3. Internal Gross Signs**

Internally, visceral mesenteries can show diffuse hemorrhage, the kidneys and liver can be hyperemic, swollen, and discolored, liver can have multifocal hemorrhages and hemorrhages can occur in the skeletal muscle (Figure 2). In chronic or neurologic manifestation, fish may show no internal gross signs.

**4. Histopathological Changes**

Histopathological changes of VHS may be widespread in the internal organs (Al-Hussinee et al. 2011; Lovy et al. 2012). Changes occur frequently in the kidney, spleen, liver, gastro-intestinal tract, and skeletal musculature (Figures 3 – 6). In Pacific Herring infected with genotype IVa, viral tropism during early infection was heavily directed at dermal fibroblasts, fibroblasts near the cartilage at the base of the fins, and endothelial cells (Figure 3). Presumably, the endothelial tropism contributes to dissemination of the virus within the internal organs (Lovy et al. 2012). Hemorrhages in the skeletal muscle and other internal organs are the result of damage to the endothelium. The kidney and spleen are often severely affected, with the hematopoietic tissue being the principal site of viral replication. Extensive necrosis, pyknosis and karyolysis of the hematopoietic cells of these organs occur (Figures 4 & 5). Renal tubules may contain cells and cellular fragments. Multi-focal hepatocellular necrosis, and diffuse necrosis of submucosal cells of the gastro-intestinal tract can occur (Figure 6). During neurologic manifestation, degeneration of peripheral nerves and optic nerves may occur. In fish with neurologic disease, the virus may be cleared from the internal organs and persist in the brain and nerves. Often brain and nerves do not show histologic changes; however, the virus proteins can be visualized by immunohistochemical staining indicating active viral replication in these sites (Figure 6; refer to further description in Lovy et al. (2012)).

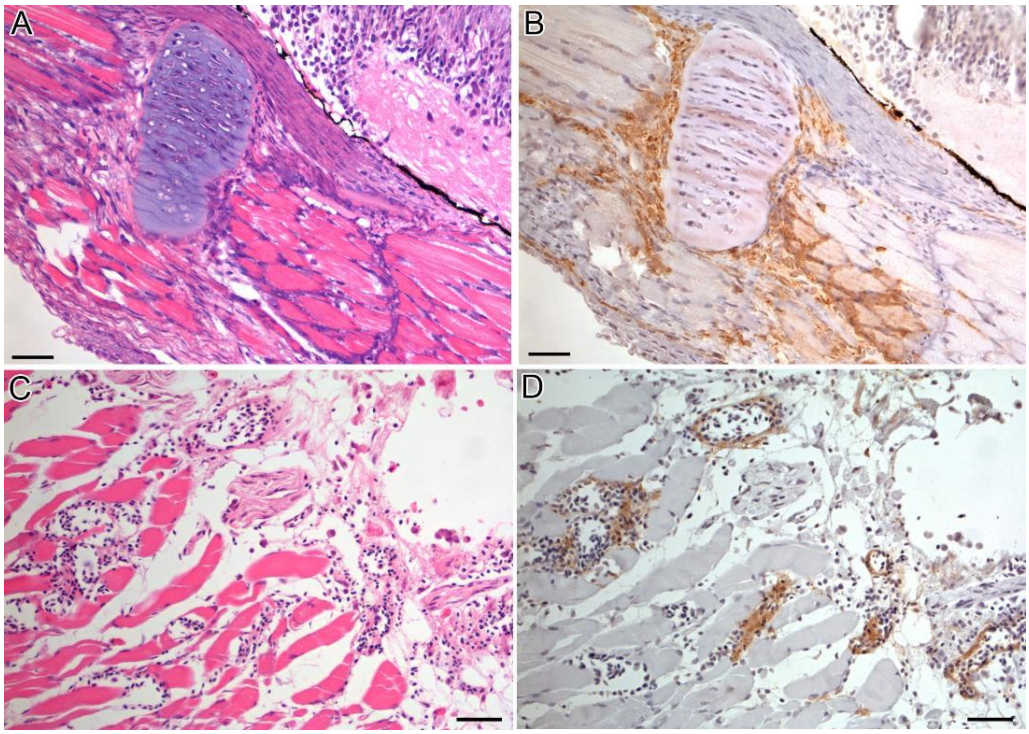


**Figure 3.** Pacific Herring with VHSV IVa. (A and B) The base of the fin with VHSV in fibroblasts surrounding the cartilage. (C and D) Blood vessels within the gut submucosa with endothelial necrosis which stains positive for VHSV. H&E staining in (A) and (C), and correlative sections with immunohistochemical staining for VHSV (golden-brown staining) in (B) and (D), respectively. Magnification bars = 20 µm.

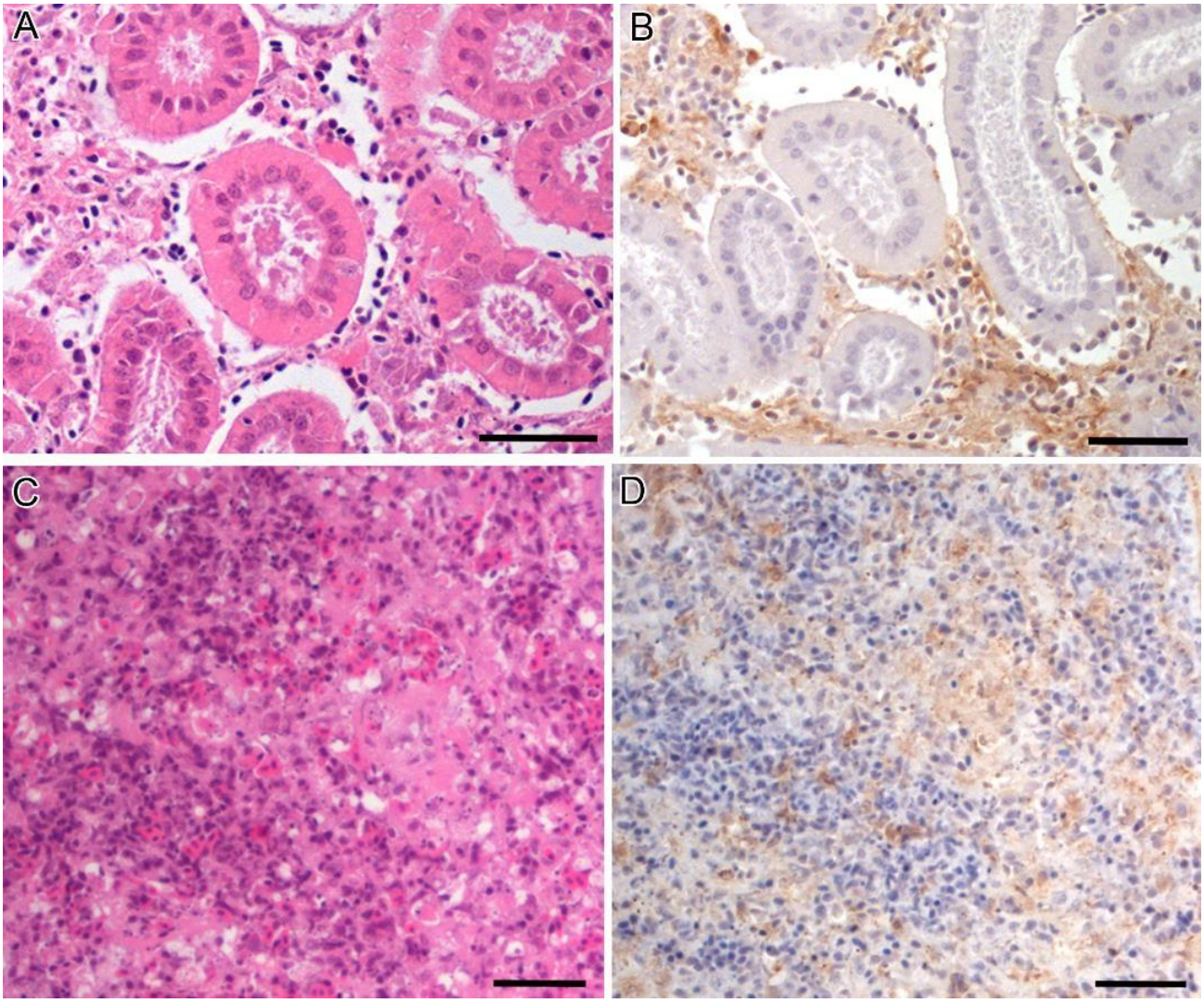


**Figure 4.** Pacific Herring with VHSV genotype IVa. Necrosis of the hematopoietic tissue and widespread staining for VHSV in the kidney (A and B) and the spleen (C and D). Staining with H&E in (A) and (C), and correlative sections with immunohistochemical staining for VHSV (golden-brown staining) in (B) and (D), respectively. Magnification bars = 20 µm.

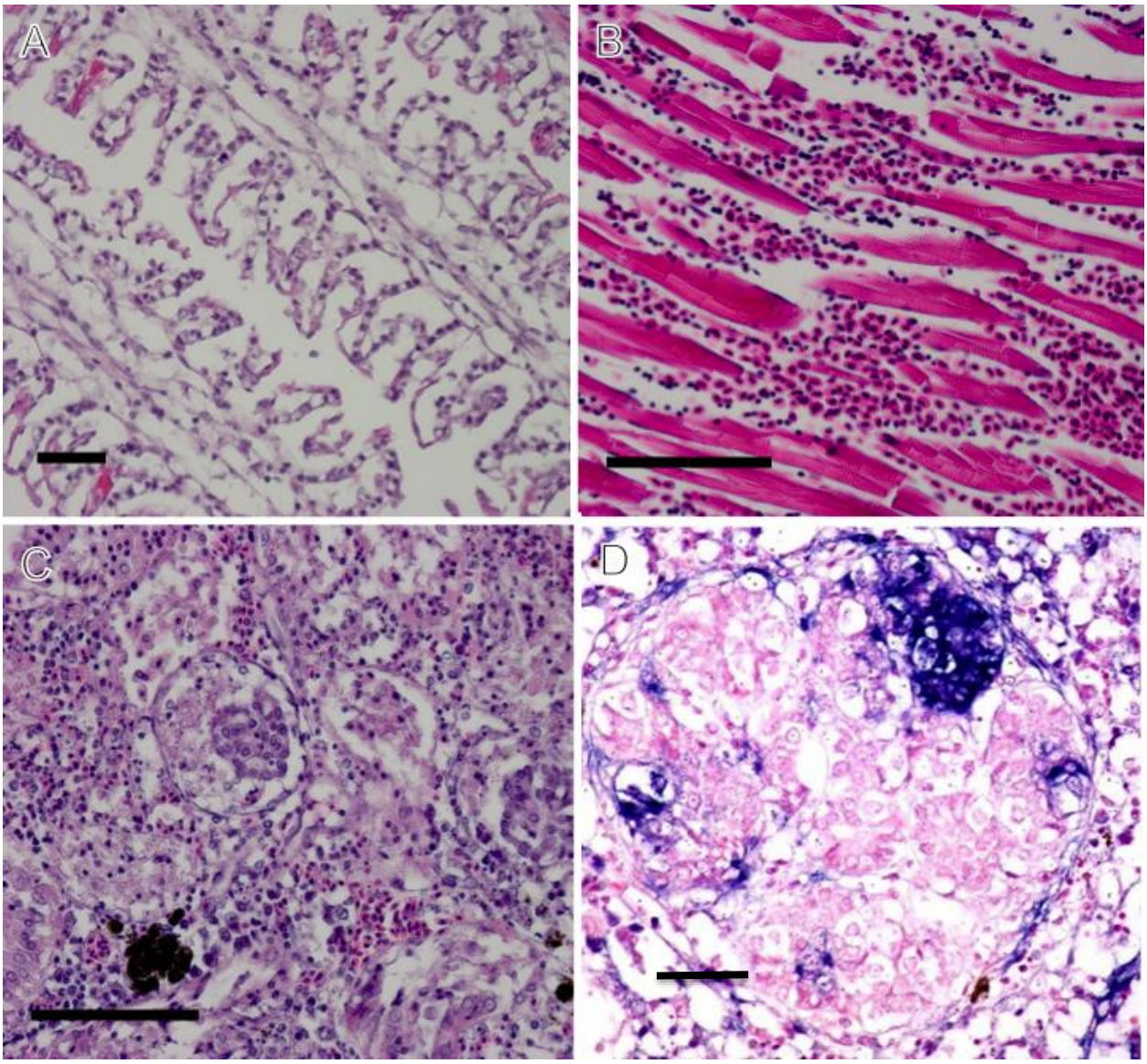


**Figure 5.** Histopathology of VHSV IVb in Muskellunge (A,B) and Yellow Perch (C,D) showing anemic gill lamellae (A), hemorrhages in the skeletal muscle (B), necrosis, pyknosis, and karyolysis of the hematopoietic cells of posterior kidney (C), and kidney glomerulus stained for VHSV (D). (A-C) H&E stain, (D) In-situ hybridization staining for VHSV. Notice the positive reaction in the endothelial lining. Magnification bar = 100 µm).

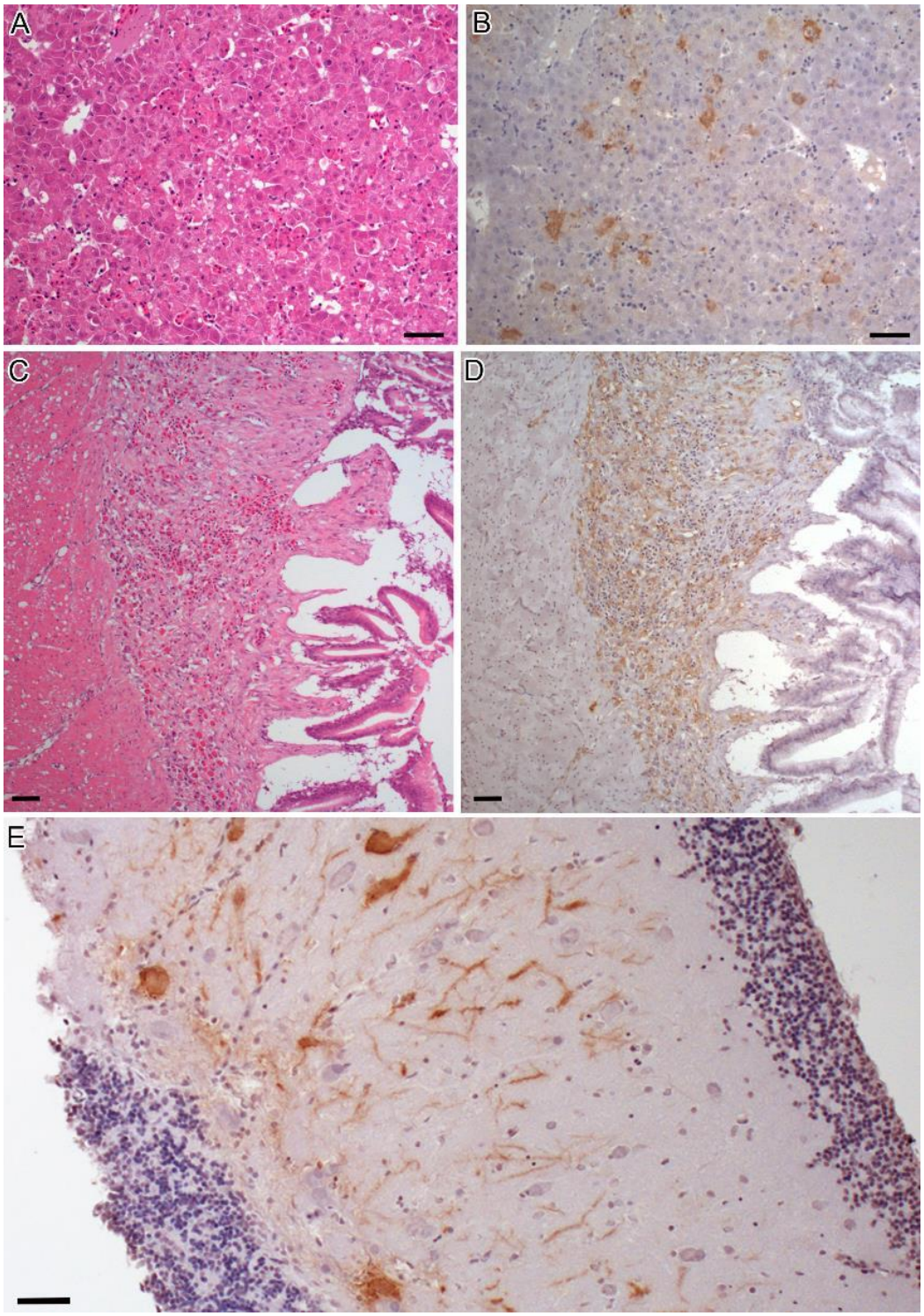


**Figure 6.** Pacific Herring with VHSV IVa. (A and B) Liver with multi-focal hepatocellular necrosis with hepatocytes in affected regions showing specific staining for VHSV. (C and D) Intestinal wall showing widespread necrosis of the submucosal tissue which correlates with VHSV-positive staining. (E) Brain from a herring with neurologic disease showing VHSV-specific staining in neurons. Staining with H&E in (A) and (C), and with immunohistochemical staining for VHSV (golden-brown staining) in (B), (D) and (E). Magnification bars = 20 µm.

# E. Disease Diagnostic Procedures

## 1. Presumptive Diagnosis

Clinical signs and histopathological changes associated with VHS, as described in section D and figures 1-6, can aid in identifying VHSV as a possible causative agent. It must be noted that disease signs and histology are variable and cannot be used for definitive diagnosis or to distinguish VHS from the other fish viral diseases. Additionally, the absence of clinical signs does not indicate that the fish are free from VHSV. Consequently, virological examination using a cell culture assay and/or molecular detection via PCR is required for diagnosis of VHSV, as further described below. VHSV is a notifiable disease to the OIE, thus after presumptively identifying VHSV, the result should be reported immediately to the appropriate regulatory agency. Figure 7 summarizes testing flow for presumptive and confirmatory diagnostic assays.

For cell culture assays, *Epithelioma papulosum cyprini* (EPC), Fathead Minnow (FHM) and Bluegill Fry (BF-2) cell lines are recommended as they are sensitive for the detection of VHSV. The Bluegill Fry (BF-2) cell line will detect all genotypes and is most sensitive for detection of genotypes I-III (Lorenzen et al. 1999; Nishizawa et al. 2006), whereas the EPC cell line has been shown to be highly sensitive for genotype IV (USGS, 2007). Cytopathic effects (CPE) in the EPC cell line are demonstrated in Figure 8. During incubation, it is critically important that the pH of the medium remain within the range of 7.4 - 7.8 because it has been suggested that the glycoprotein of VHSV undergoes a pH-dependent conformational change that can prevent development of cytopathic effect (CPE) in acidic cultures (Gaudin et al. 1995). This is especially problematic for cell lines derived from coolwater species that continue to metabolize efficiently at the incubation temperatures of the assay. For diagnostic cases of clinical fish suspected for VHS, samples should be processed independently and not pooled with other fish samples. When screening asymptomatic fish, pooling tissue samples from up to 5 fish is common.

Various polymerase chain reaction (PCR) assays have been validated for presumptive detection/diagnosis of VHSV, of which three are widely used (Garver et al. 2011; Jonstrup et al. 2013; Kim et al. 2018). These assays may be utilized to obtain reliable results with a fast turn-around compared to using viral cell culture assays. Two of these are TaqMan-based real-time reverse transcription PCR (rRT-PCR) assays targeting the N-gene and are sensitive for detection of all genotypes; one includes a separate step to generate complementary DNA (cDNA) (Garver et al. 2011), which is advantageous for sample storage since DNA is more stable than RNA. The other is a one-step assay (Jonstrup et al. 2013) that has the reverse transcription step built into the PCR, reducing the number of pipetting steps in the assay. Comparison of various rRT-PCR assays across multiple laboratories in the USA has demonstrated that the Jonstrup et al. (2013) assay produced the most consistent analytical performance for diagnosis of all VHSV genotypes (Warg et al. 2014a) and had high sensitivity and specificity (Warg et al. 2014b). The Jonstrup et al. (2013) assay has at least comparable diagnostic sensitivity to cell culture methods (Jonstrup et al. 2013; Warg et al. 2014b). A third assay by Kim et al. (2018) is a conventional PCR (RT-PCR) also targeting the N-gene and, similar to the two assays above, has been well validated according to the OIE standards. Section 2 of the Inspection manual herein has adopted the use of the one-step rRT-PCR assay by Jonstrup et al. (2013). See further details in Section 2 of this manual. The three aforementioned PCR assays may be used to screen asymptomatic fish or fish showing clinical disease signs. Similar to viral cell culture assays, fish exhibiting clinical signs in diagnostic cases should not be pooled, whereas screening of asymptomatic fish may be pooled with up to 5 fish. When using 5-fish pools, tissue processing protocols similar to those employed in virus isolation assays should be used. If negative results are obtained using these molecular assays, then these samples are considered negative and no further testing is necessary. If a positive result is obtained, then further work utilizing a second independent

assay is required to confirm this finding (see below and Figure 7).

**2. Confirmatory Diagnosis**

Following the observance of CPE in cell culture consistent with VHSV, confirmatory testing must be performed. Molecular assays, including either conventional or rRT-PCR, have been extensively validated for use in confirmation of VHSV (Garver et al. 2011; Jonstrup et al. 2013; Warg et al. 2014a; Warg et al. 2014b; Kim et al. 2018). Additionally, determination of genotype can be done by sequencing of the PCR amplicon, thus conventional PCR is ideally utilized for identification of VHSV. A conventional PCR described by Hedrick et al. (2003) targets the G-gene, which is most informative for genotyping. Protocols for sequence analysis are further detailed (Hedrick et al. 2003; Snow et al. 2004; Garver et al. 2013). Other, less commonly used methods also exist for confirming the virus, including a serum-based virus neutralization assay (Millard and Faisal, 2012a,b), immunoblot assay (McAllister and Schill 1986; McAllister and Owens 1987), enzyme-linked immunosorbent assay (ELISA; Way and Dixon 1988; Olesen and Jorgensen 1991; Mourton et al. 1992; Faisal et al, 2019), fluorescent antibody test (FAT; Lorenzen et al. 1988), and DNA probe (Batts et al. 1993). For the virus neutralization assay, the cell cultures and the conditions of incubation and pH control must be maintained as indicated above. Antiserum specific to each serotype must be used in the virus neutralization assay because three serological types of VHSV can be distinguished by certain neutralizing antisera. For the ELISA and FAT, polyclonal or monoclonal antibodies are available that react with all VHSV serotypes.

If a presumptive VHSV PCR positive sample is negative using a different confirmatory PCR assay, then a single repeat of testing on the sample should be conducted. The repeat test should include re-extraction of RNA from the testing sample. Both RNA samples, the original RNA extracted and the RNA from the repeat extraction should be evaluated. If negative after the repeat run, the presumptive assay should be repeated to confirm that it is in fact positive. If the original presumptive test has been confirmed as positive, then this test result should be reported out as suspect, and further sampling of the population may be warranted. If both the presumptive and confirmatory tests are negative when repeated, then the sample should be reported as negative. If a presumptive positive detection of VHSV occurred by using PCR on tissues from wild fish from known VHSV-positive zones, then confirmation may be done using a conventional RT-PCR assay, that detects all genotypes of VHSV and is independent from the initial PCR, such as those described by Hedrick et al. (2003) and Kim et al. (2018), and sequence analysis to confirm the genotype (Hedrick et al. 2003; Snow et al. 2004; Garver et al. 2013). If a presumptive positive detection of VHSV occurred by using rRT-PCR or the conventional PCR (Kim et al. 2018) in farmed fish or wild fish collected from a non-VHSV endemic zone, then this suspected positive result must be confirmed by virus isolation in cell culture. Confirmed isolates of VHSV from a new host species or a new geographic area should be sent to a reference laboratory (e.g., the NVSL) for additional confirmation and the finding reported to state or national fisheries agencies. For confirmation in a reference laboratory, it is recommended to send a duplicate tissue sample, which has not been previously extracted or processed.

Phylogenetic analysis of viral gene nucleotide sequences utilized to genotype VHSV provides data for epidemiological studies that may lead to better understanding on the origin and/or an extension of the range of a particular genotype and ultimately better pathogen control. Sequence analysis and genotyping may be conducted at a research or diagnostic laboratory or sent to a reference lab. It is worthy of noting that the reference laboratory will sequence the virus as part of the confirmation process, thus sequencing may not be necessary by individual testing labs. For the confirmatory diagnosis, it is recommended to amplify and sequence the G-gene, since to date this is the most informative gene for genotyping and abundant data exists for this gene from other viral sequences for comparison. Primers for amplification of the G-gene and cycling conditions for conventional PCR for the North American genotype (IV) have been established (Hedrick et al. 2003; Garver et al.

2013). A nested PCR can be utilized to obtain the central region of the G-gene (Hedrick et al. 2003) and other primer combinations can yield the entire G-gene (Garver et al. 2013) for the most complete sequence and resolution of the genotype. Direct sequencing may be done from the PCR amplicon.

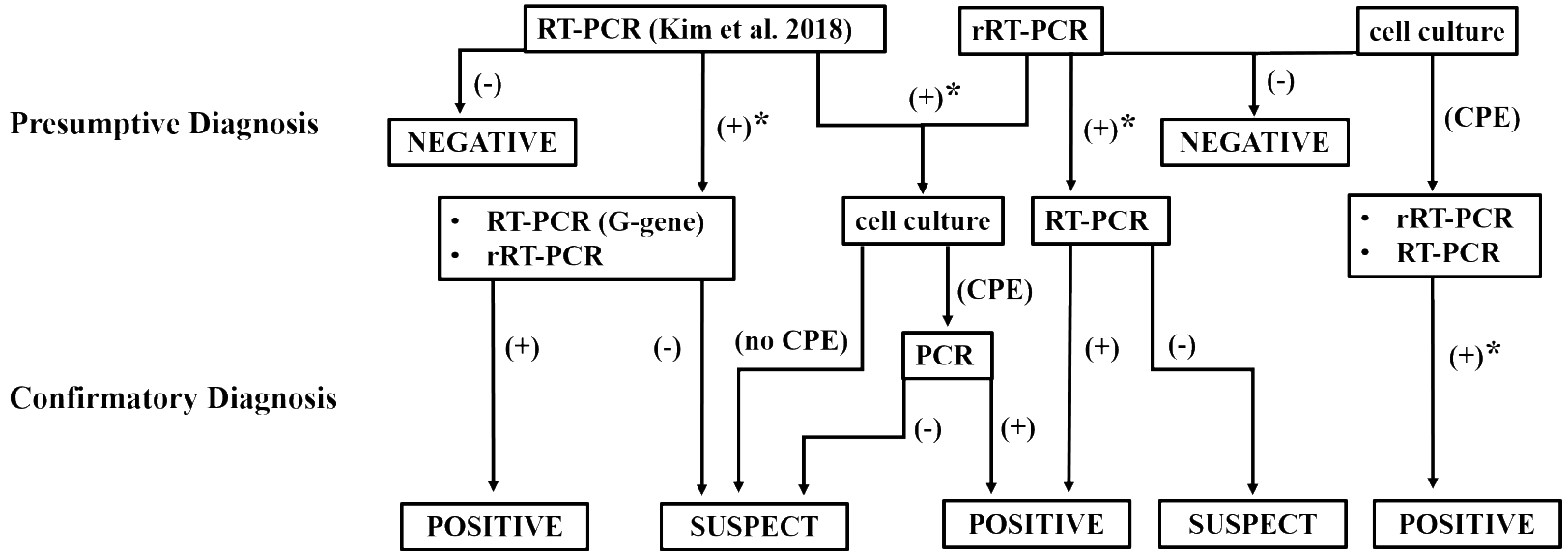


**Figure 7. Typical testing scheme for diagnosing VHSV.** When VHSV is suspected (*), then this should be reported immediately to a regulatory authority. Confirming VHSV requires two independent positive assays. POSITIVE samples should be sequenced and genotyped. If confirmatory tests are negative, samples are SUSPECT and reported as such unless additional action is taken, such as retesting, resampling or use of an alternative assay.

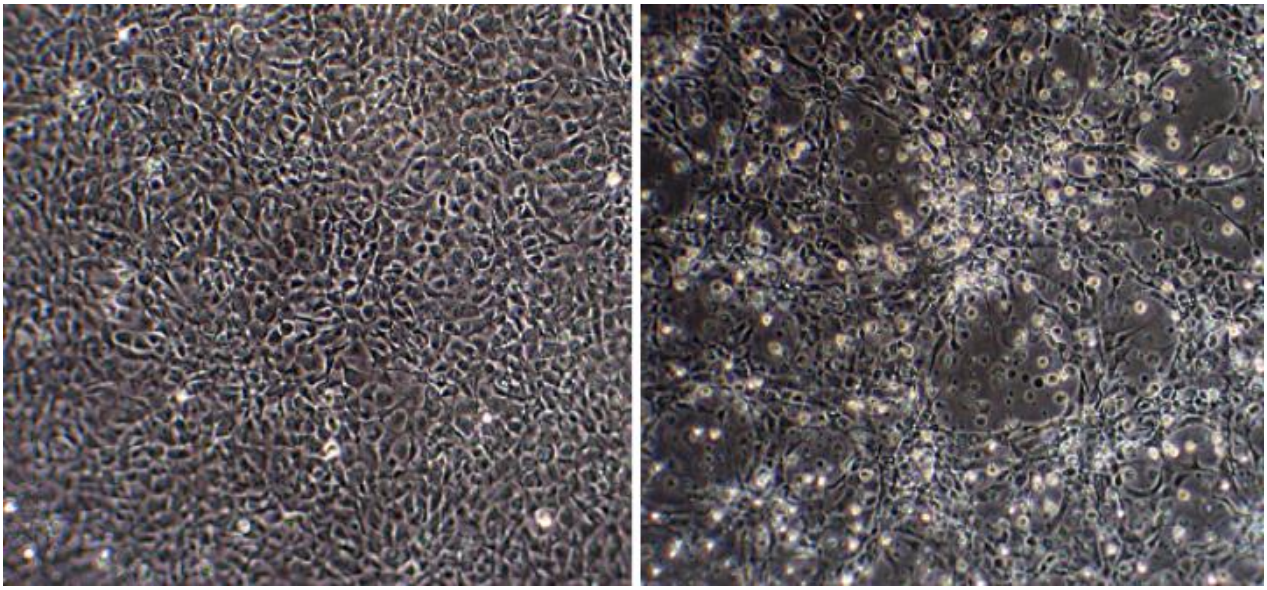

**Figure 8.** VHSV induced cytopathic effect (CPE) on the EPC cell line. (A) Negative control cells. (B) 48 hours post-inoculation. Notice cell rounding and lysis of cell sheet.

## F. Procedures for Detecting Subclinical Infections

Subclinical infections can be detected by cell culture assay, rRT-PCR (Garver et al. 2011; Jonstrup et al. 2013) or conventional RT-PCR (Kim et al. 2018). In some instances, VHSV has only been detected by examination of certain tissues or organs such as the brain. Because the virus has been shown to persist in brain and nerves for extended periods, brain should be included in the sample, especially when no clinical disease signs are apparent. Follow the procedures outlined above for presumptive and confirmatory diagnosis. Detection of VHSV by rRT-PCR (Garver et al. 2011; Jonstrup et al. 2013) on RNA extracted directly from tissue homogenates prepared following typical virus isolation tissue processing protocols on five-fish pools is as sensitive as isolation of VHSV in cell culture (Garver et al. 2011; Jonstrup et al. 2013). However, the impact of pooling fish samples on any of the VHSV assay's diagnostic sensitivity has not been well studied, yet it is typical to process samples in five-fish pools for virus isolation. When low viral concentrations would be expected in samples, i.e. when screening fish populations not exhibiting clinical signs and/or looking at large fish, it would be beneficial to test individual fish instead of pooling samples. It is possible that pooling fish will dilute individual samples to undetectable levels. Brain and kidney pools from individual fish would be an ideal sample when screening for subclinical infections.

## G. Procedures for Determining Prior Exposure to the Etiological Agent

The specific immune response among survivors of VHS epizootics and unapparent virus carriers varies with both the fish and season of the year. Nevertheless, detection of VHSV-specific antibody can be useful as part of a VHSV surveillance program for evidence of past infections (Millard and Faisal 2012a,b; Millard et al. 2014, Faisal et al. 2019) and for determining the status of the protective immune response following vaccination (Bernard et al. 1983; Olesen and Jorgensen 1986, Standish et al. 2016; Standish and Faisal 2017).

## H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Tissue storage varies according to the testing protocols to be utilized. Optimal tissue samples should be maintained on ice, preferably below 4°C, but not frozen. Tissue samples should be submerged in transport medium, preferably cell culture medium (pH 7.4 - 7.8) with added antibiotics. The OIE (2019) recommends the combination of 200 International Units (IU) penicillin, 200 μg streptomycin, and 200 μg kanamycin per ml of transport medium. Samples should be processed within 48 h.

Freezing of samples may be appropriate in certain testing regimes. Whole fish or tissue samples may be frozen immediately following collection and maintained frozen at -80°C until further processing. If only PCR assays are to be utilized and immediate freezing or maintaining frozen samples is not possible, then the tissue may be preserved in an RNA preservative. Tissue in RNA preservative is not suitable for cell culture assays. Frozen tissues are suitable for evaluation by RT-PCR or rRT-PCR. Freezing is known to reduce infectivity of the virus (Arkush et al. 2006; Phelps et al. 2013), though the virus can still be isolated in cell culture after freezing, particularly from clinical fish with heavy concentrations of virus. Thus, frozen samples from clinical fish that are expected to have higher concentrations of virus should still yield a high likelihood for isolation of the virus in cell culture. Considering the potential for reduced viral infectivity following freezing, it is suspected that sensitivity of cell culture will be reduced in frozen samples. Samples should not be stored in glycerol because VHS virus has been shown to be inactivated using this method.

## I. Procedures for Enumeration of VHSV

The EPC cell line is recommended for enumeration of VHSV infectious units via a plaque assay or TCID50 assay. Virus adsorption to EPC cells can be enhanced by pretreating the cells with a 7% solution (final concentration) of polyethylene glycol (PEG: 20,000 MW (Sigma Aldrich Catalog # P-2263); Batts and Winton 1989) or by adding DEAE dextran (Campbell and Wolf 1969; 50 μg/mL final concentration). Quantitative rRT-PCR assays have been developed that can be used to estimate virus genome loads for some (Chico et al. 2006; Hope et al. 2010) or all strains of VHSV (Garver et al. 2011; Jonstrup et al. 2013).

## J. Procedures for Determination of Disease-free Status

Inspection procedures for determination of disease-free status rely upon negative findings on viral assays using cell culture or PCR. Viral assays using cell culture are ideal for conducting fish health inspections, as cell lines detect active replication of numerous viruses of concern as well as previously unknown agents. Molecular detection by rRT-PCR (Garver et al. 2011; Jonstrup et al. 2013) or by RT-PCR (Kim et al. 2018) has been recognized as equivalent in sensitivity as viral cell culture assays. All genotypes of the virus can be detected using these methods, and all have been well validated according to the OIE (2019). Negative results with use of any of these assays are considered negative, and any positive results must be confirmed as outlined above.

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