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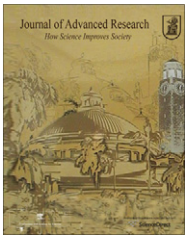

REVIEW

Emergence and resurgence of the viral hemorrhagic septicemia virus (*Novirhabdovirus*, *Rhabdoviridae*, *Mononegavirales*)

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Abstract Viral hemorrhagic septicemia virus (VHSV) is one of the most serious pathogens of finfish worldwide in terms of its wide host-range, pathogenicity, disease course, and mortality rates. The disease was first documented in the 1930s in Europe in association with heavy losses in rainbow. Data collected over 50 years show that VHSV is a virus of marine origin: its ability to alternate between marine and freshwater environments remains an enigma which requires further investigation. In 2003, VHSV invaded the Laurentian Great Lakes basin causing devastating losses. VHSV is believed to have negative impacts on a number of important Atlantic, Pacific, and Great Lakes fish species. Phylogenetic analysis has offered clues into the geographic and host range of the virus, but sporadic outbreaks of the disease in uncommon locations have imparted unforeseen challenges in delineating the virus' regional distribution. The virus' ability to gain access to aquaculture farms

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has also positioned it as a disease of utmost priority once detected in these settings. Current diagnostic methods, while greatly improved, are hampered by the variability of disease course among susceptible species. In general, VHSV causes severe degeneration in the hematopoietic tissues of affected fish. Based on historical and current data, it is feared that VHSV will continue to mutate, expand to other geographic areas, and infect new host species. As a result, immediate international attention and coordination of efforts are needed. The objective of this review article is to provide an updated synopsis on the current status of VHSV epizootiology and pathogenicity.

Historical background

Viral hemorrhagic septicemia virus (VHSV) is one of the most pathogenic viral diseases of finfish worldwide [1]. The disease was first recognized as early as 1938 when Danish rainbow trout farms became stricken with what Schäperclaus described as "infectious kidney swelling and liver degeneration" [2]. A multitude of outbreaks were soon documented that were associated with heavy economic losses [3]. A viral etiology was strongly suspected by Schäperclaus [4] because the disease was transmitted using bacteria-free filtrates of tissue homogenates from affected fish. Within three decades since the initial report, the viral etiology was confirmed by Jensen in 1963 when he isolated the virus on a rainbow trout gonad cell line (RTG-2) [5], and promptly described it as "Egtved" virus, but the disease was renamed as Viral Hemorrhagic Septicemia by the Office of International Epizooties currently known as the World Animal Health Organization [6]. This terminology remained until 2000 when the International Committee for Taxonomy of Viruses changed the name of the virus into VHSV [7]. The virus was then isolated from a number of other European countries such as France, Italy and Poland [8–11].

By the 1970s, researchers embarked on a series of studies attempting to characterize and elucidate the biological and biophysical aspects of the virus [12–17]. Serologic investigations revealed the presence of two distinct strains of VHSV [18,19], but isolates obtained from brown trout later revealed the presence of a third strain [20]. The discovery that a virus was responsible for mass mortalities in cultured rainbow trout prompted many investigations in Europe to characterize the newly isolated deadly virus [21] and to develop sensitive diagnostic assays [19]. Moreover, Koch's postulates were fulfilled through a series of experimental infection studies [22,23]. By the late 1970s, it was believed that the "Egtved" virus was strictly a disease of rainbow trout farmed in freshwater.

Until the late 1980s, the geographic and host range of VHSV was believed to be confined to the waters of continental Europe [1], but Hopper [24] and Brunson's [25] discovery of VHSV in spawning chinook salmon (*Oncorhynchus tshawytscha*) at the Glenwood Springs Hatchery on Orcas Island, Washington State (WA), USA and from spawning coho salmon (*Oncorhynchus kisutch*) at the Makah National Fish Hatchery, WA confirmed the expansion of VHSV range to involve North America. The following year, two more isolations occurred in wild adult coho salmon from Lummi Bay Hatchery [26] and Soleduck Hatchery in WA [27]. This surprise prompted North American regulatory agencies to implement health inspections and surveillance of salmonids and other fish species. By 1990–1993, VHSV was isolated from sport caught Pacific cod (*Gadus macrocephalus*) in Prince William Sound (PWS), Alaska [28–30]. During this same time frame, VHSV continued to devastate freshwater and marine waters in Europe. Simultaneous reports of VHSV outbreaks were described in another marine fish; the turbot (*Scophthalmus maximus*) farmed in the Baltic Sea [31]. These findings raised the suspicion that VHSV was of marine origin.

In 1993, adult Pacific herring (*Clupea harengus pallasi*) returning to spawn in PWS were found positive for VHSV [30]. Meyers and Winton [32] raised the suspicion that VHSV may negatively impact Pacific herring at the population level as it affected all age classes. Additional VHSV positive Pacific herring were sampled from British Columbia, Canada and Puget Sound, WA, which suggested that this particular strain of VHSV was spreading regionally and that it may have become enzootic in Pacific herring populations from Alaska to Washington [32]. Then in 1999, VHSV was isolated from a mass mortality episode that involved multiple species in Alaska such as the Pacific herring, Pacific hake (*Merluccius productus*) and walleye pollock (*Theragra chalcomgramma*) [33], thereby providing evidence that VHSV was more widespread in the marine environment than previously thought. Such discoveries in the coastal waters of Alaska and the Pacific Northwest led to a study conducted by Hedrick et al. [34] which revealed the presence of VHSV in sardine (*Sardinops sagax*), mackerel (*Scomber japonicus*), Pacific herring, shiner perch (*Cymatogaster aggregata*), eulachon (*Thaleichthys pacificus*), sablefish (*Anoplopoma fimbria*) and surf smelt (*Hypomesus pretiosus pretiosus*) sampled from British Columbia, Canada to the southern California coast.

The North American VHSV isolations ignited further testing of marine fish populations in European waters. Such marine surveys revealed the presence of VHSV in new geographic areas and infecting new hosts such as farmed turbot off the west coast of Scotland [35], Atlantic cod in the North Sea [36], Atlantic herring (*Clupea harengus*) from the English Channel [37], haddock (*Melanogrammus aeglefinus*) and sprat (*Sprattus sprattus*), blue whiting (*Micromesistus poutassou*), whiting (*Merlangius merlangus*), and lesser argentine (*Argentina sphyraena*) throughout the Baltic Sea, Kattegat, Skagerrak, and North Sea [38]. In addition to Europe and North America, VHSV has also been isolated in Japan from wild Japanese flounder (*Paralichthys olivaceus*) in Wakasa Bay of the western Pacific Ocean [39,40] in 1999 and in Korea [41]. Based on the information gathered from all over the world, by the late 1990s, a consensus started building that VHSV primarily affects marine fish and that VHSV may have a marine origin [42].

The expansive and highly adaptable nature of VHSV was clearly underestimated after being isolated from adult muskellunge (*Esox masquinongy*) sampled from Lake St. Clair, Michigan between 2003 and 2005 [43], at which time no other rhabdoviruses were known to infect any fish species in the

Great Lakes Basin. This initial discovery was soon followed by reports detailing the isolations from mass mortality events in the freshwater drum (*Aplodinotus grunniens*) [44] in the Bay of Quinte, Lake Ontario, Canada in 2005, round gobies (*Neogobius melanostomus*) [45] and mummichog (*Fundulus heteroclitus heteroclitus*), three spined stickleback (*Gasterosteus aculeatus*), striped bass (*Morone saxatilis*), and brown trout (*Salmo trutta*) in eastern Canada sampled between 2000 and 2004 [46]. Other documented species from which VHSV has been isolated from throughout the Great Lakes Basin include gizzard shad (*Dorosoma cepedianum*), yellow perch (*Perca flavescens*), northern pike (*Esox lucius*), shorthead redhorse sucker (*Moxostoma macrolepidotum*), silver redhorse sucker (*M. anisurum*), rock bass (*Ambloplites rupestris*), emerald shiner (*Notropis atherinoides*), spottail shiner (*N. hudsonius*), crappie (*Pomoxis nigromaculatus*), bluegill (*Lepomis macrochirus*), pumpkinseed (*L. gibbosus*), chinook salmon, lake whitefish (*Coregonus clupeaformis*), walleye (*Sander vitreus*), and largemouth bass (*Micropterus salmoides*) (unpublished data – Aquatic Animal Health Laboratory, Michigan State University). Currently, the VHSV-related events in the Great Lakes Basin have emphasized the need to study the origin of VHSV and its ability to devastate fishes in both the freshwater and marine environments and the path it takes to spread.

More recently VHSV was isolated from rainbow trout in the United Kingdom [47]. While this resurgence of VHSV in Europe is not surprising, the isolation reported by Stone et al. [47] demonstrated the first VHSV isolation in the United Kingdom (UK) was obtained from a freshwater species in a freshwater environment. The investigators attributed the outbreak to a nearby stream that may have been harboring the virus for a long period of time. The results of this report emphasized the fact that while legislation, biosecurity, and sound management practices can greatly reduce the spread of VHSV, the virus' covert nature enables it to continue expanding into new geographic locales. The re-emergence of VHSV was also experienced in Norway in seawater-farmed rainbow trout [48]. The authors concluded that the outbreak was most likely a result of viral transmission from the marine environment. Both isolations have thus confirmed VHSV's capacity to emerge and resurge in the northern hemisphere.

Virus characterization

VHSV is a member of the order, *Mononegavirales*, family *Rhabdoviridae* and genus *Novirhabdovirus* [7], which belongs to a group of bullet shaped viruses that measure approximately 70 nm in width by 180 nm in length. An envelope, which contains the major surface antigen, glycoprotein, encases the virus and is believed to be important for virus adsorption and attachment to susceptible cells [49]. Four structural proteins are present, along with an RNA dependent RNA polymerase [21,49–51]. The VHSV genome is a single, negative-stranded ribonucleic acid (RNA) and is approximately 11–12 kb containing six open reading frames [52] in the following order; nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonstructural protein (NV) and lastly the viral RNA polymerase (L) (Fig. 1). Translated proteins have been measured with molecular masses as follows: 38–41 kDa (N), 21.5–25 kDa (P), 19 kDa (M), 72–80 kDa (G), and 157–190 kDa (L) [21,53].

Information regarding the gene functions of VHSV are largely provided by studies conducted on mammalian or other fish rhabdoviruses. The glycoprotein gene is particularly important in that it is the primary neutralizing surface antigen of VHSV and other rhabdoviruses [54,55]. The nucleoprotein is responsible for tightly enclosing the RNA genome thereby creating the ribonucleoprotein core [55]. Both the glycoprotein and nucleoprotein genes have enabled investigators to conduct phylogenetic studies. The phosphoprotein, also known as the internal matrix protein is considered as the "viral transcriptase" when associated with the L and P proteins [56]. The M protein acts as a bridge between the viral envelope and nucleocapsid [55], but is also said to play a regulatory role in viral transcription, replication, production, and budding [57–59].

Disease

Tissue pathogenesis

The initial emergence of VHSV in rainbow trout farms led to a wealth of information about VHSV-associated clinical signs and histopathologic alterations in spontaneously and experimentally infected rainbow trout. Clinical signs, although non-specific can appear singly or in a combination of either dermal petechial hemorrhages, hemorrhages at the base of fins, severely pale gills, periocular hemorrhage, lethargy, and abnormal to erratic swimming [60]. These findings have been reproduced through experimental infection [61] (Fig. 2a–d).

In the rainbow trout, endothelial cells of the kidney and spleen are the first targets of the virus within the first 1–4 days of infection [23,62–64]. Soon thereafter the hematopoietic elements of the kidney and spleen tissue undergo necrosis and degeneration, most notably at melanomacrophage centers [64]. Immunohistochemistry has previously revealed that VHSV can be detected in macrophages and melanomacrophages, supporting the claim that replication occurs in these cells during both acute and chronic infection [65–67]. By days 4–7 after experimental infection, multifocal degeneration of the liver can be observed in histopathologic specimens [61,23,63,64]. While conflicting data exist whether the liver is considered a primary target of the virus, disease course studies have collectively supported the idea that endothelial cells of the liver do not appear to be affected owing to the idea that liver endothelial cells may lack the appropriate receptors for viral attachment [63,64].

Forms of disease

Spontaneous and experimental infection studies have demonstrated acute, subacute, and chronic forms of the disease. In some hosts, the chronic form is characterized by nervous manifestations [1,49]. The clinical and associated pathologies

Fig. 1 Viral hemorrhagic septicemia virus genome comprised of six open reading frames and a total length of 11,158 bases.

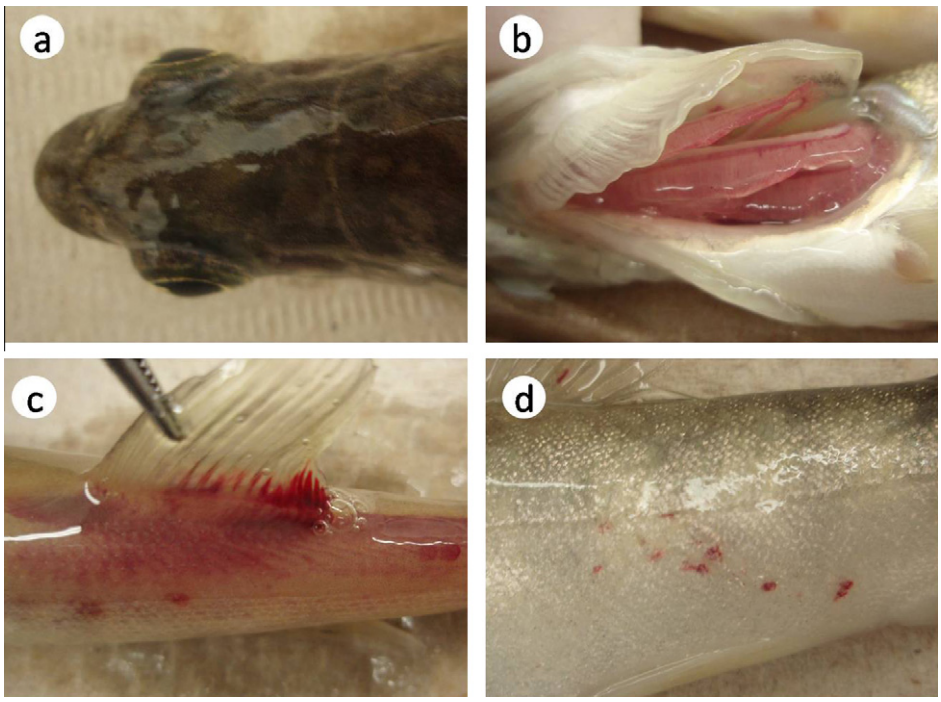

Fig. 2 (a) Lake trout (*Salvelinus namaycush*) showing moderate exophthalmia, (b) severe gill palor (c), hemorrhaging at the base of the dorsal fin of a juvenile muskellunge (d), and petechial hemorrhages in the epidermis of a splake (*Salvelinus namaycush* × *S. fontinalis*).

vary with disease form, with the acute disease being the most rapid and resulting in greater mortality. Chronically infected fish experience a prolonged course with lingering low level mortality and often resulting in fish that are virus carriers, which shed VHSV virus particles into the surrounding environment [68]. The nervous form is often associated with erratic swimming behavior characterized as spiraling and/or flashing [60].

Transmission

Lateral transmission is the primary method by which VHSV infects new hosts. Fish that are chronically infected often become carriers and shedders of the virus. Once the virus has been shed into the water through urine, the virus attaches to the gill epithelium and/or skin of nearby hosts [67]. Whether the virus enters the host immediately or replicates at the attachment for an extended period of time before entry is unclear, however viral replication has been shown to occur in both the skin and gills [69]. More specifically, viral replication has been observed in gill pilar cells [70,71], which suggest the gills may not serve merely as a portal of viral entry [62]. When gill epithelia of rainbow trout were cultured and infected with VHSV isolates from the marine and freshwater environment, investigators discovered that the predilection of certain VHSV isolates for gill tissues depends on the origin of virus and fish species, respectively [72]. While several studies have indicated the gills as a major site of entry, a recent study conducted by Harmache et al. [73] also showed the fin base as a site of viral entry through a noninvasive bioluminescence assay. The skin has also been proposed as an entry site for virus but experimental data have revealed that VHSV can be detected after fish have become viremic [64,69].

The transmission of VHSV from fish to fish through urine as the only means has also been challenged. Traditional insights into viral pathogenesis strongly suggest this pathway as the only means of transmitting virus. Oral transmission has been demonstrated [74] by the feeding of infected fish. Investigators have also proposed that leeches [75] could serve as mechanical vectors, yet additional studies are required to determine if VHSV replication takes place in the leech internal organs. Furthermore, fish-eating birds have been proposed as possible vectors, but only if the fish has been regurgitated to a different body of water. Fecal transmission in birds has not been strongly suspected given the acidity of digestive secretions and the bird's body temperature which are not ideal for VHSV. While these assumptions have yet to be verified, the source of outbreaks is all too often attributed to covertly infected fish or transfer from the surrounding environment. Therefore the need to clarify the relationships between the virus and environment requires further study.

Experimental studies have also provided insights into how the virus can be transmitted. One of the most common methods utilized by investigators has been through waterborne challenge, which mimics a natural course of infection [76–94]. The method involves immersion of a group of fish into a known concentration of virus infected water, usually expressed as plaque forming units (pfu/mL) or median tissue culture infectious dose ($TCID_{50}$). Alternatively infection with VHSV has also been demonstrated by intraperitoneal injection, which allows for approximately equal amounts of virus delivered to each fish [76,77,79–87,89–94]. A third method of transmission known as cohabitation requires that VHSV carrier fish are placed inside a tank with naïve fish [94]. Although each of the transmission modes is different mechanically and

approach, all methods are capable of producing the clinical diathesis of VHSV infection.

Factors influencing disease course

A number of factors predispose fish to VHSV infection including stress, age, and water temperature. Stressors such as dramatic changes in the environment have been noted to be responsible for the development and onset of infection. Hoerlyck et al. [95] reported an instance of isolating VHSV from maricultured rainbow trout that were VHSV negative prior to transport from the freshwater environment. VHSV is considered to be a coldwater disease with an optimal temperature of 9–12 °C [49]. This is interesting considering that VHSV replicates best on cell lines at 14–15 °C [13]; however, this phenomena is not unique among aquatic pathogens. These differences may be the result of differences in the origins of VHSV isolates which may require different environmental parameters *in vitro* versus *in vivo*. The pathogenicity of VHSV is greatly diminished at or above 20 °C [13]. More recently, it was determined that VHSV can survive up to 14 days in freshwater and only 4 days in seawater [96]. Factors that also influence survivability of the virus include whether the water was filtered prior to the addition of virus and the temperature at which the virus and water mix was held. These results were partially corroborated by Kocan et al. [97], who also found that virus survival was greatly enhanced if the virus was maintained in serum enriched cell culture medium or water that contained ovarian fluid.

The age at which fish become infected also determines the course and outcome of disease. Infected fry-juvenile age fish may experience up to 100% mortality while fish that are older when infected may exhibit 25–75% mortality rates [32,98]. Experimental infections have demonstrated marked mortality rates especially in the case of juvenile herring at 5 months of age compared to 9 and 13 months of age [85]. However, Ghittino [10] determined that fish from any age group could become infected, with younger fish being more susceptible and fish greater than 6 months appearing resistant. The findings were further supported by studies conducted on rainbow trout by Roberts [99] and Bellet [8], who determined rainbow trout greater than 2–6 months to have milder cases of disease, while fish older than 2 years were almost completely resistant.

Tissue alterations caused by VHSV

Histopathologic examination of certain tissues from diseased fish has offered invaluable insights into the pathogenesis of VHSV. The most pronounced changes have been reported in the muscle, liver, spleen, kidney, and at times in the swimbladder (Fig. 3a) [60]. Despite no actual damage to muscular tissues, bleeding in organized discrete foci can often be observed between muscle bundles and fibers (Fig. 3b). Liver sinusoids are often distended along with hepatocellular vacuolation and degenerative changes. Kidney tissues can exhibit variable changes depending on the stage of infection. Yasutake reported lymphopenia to be the predominant alteration during acute infection, while hyperplastic changes are observed in chronic stages of disease [60]. When Yasutake and Rasmussen investigated histopathogenesis in experimentally infected rainbow trout, they found that the kidneys were the initial target site of VHSV, which was followed by changes in the spleen,

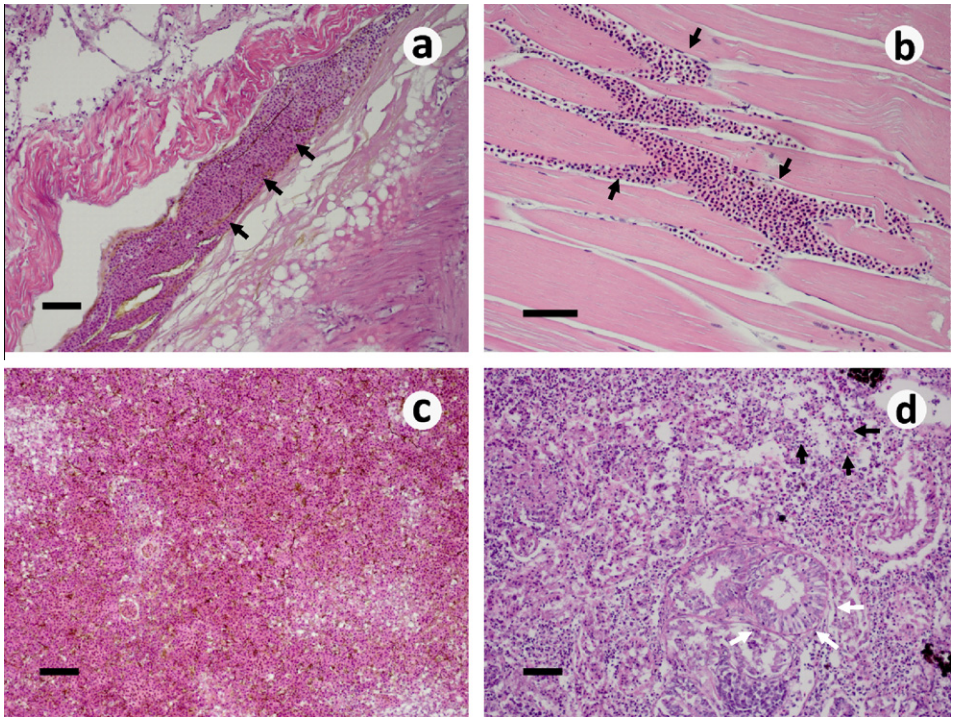

Fig. 3 (a) Tissue section of a viral haemorrhagic septicaemia infected muskellunge showing widespread hemorrhage in the swimbladder wall (scale bar = 100 μm, black arrows). (b) Infiltration of erythrocytes between muscle bundles and fibers of a lake trout (scale bar = 100 μm, black arrows). (c) Spleen section of a largemouth bass showing severe congestion (scale bar = 200 μm). (d) Tissue section of a muskellunge kidney exhibiting widespread haematopoietic depletion (black arrows) and necrosis of tubular epithelia (white arrows) (scale bar = 100 μm).

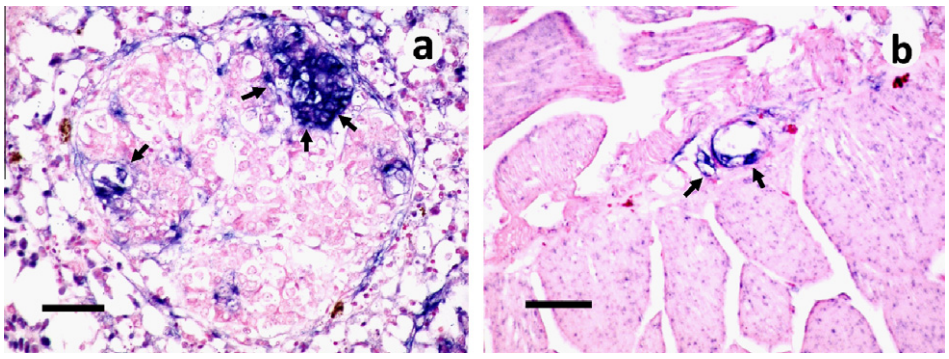

Fig. 4 (a) *In situ* hybridization of a kidney section from yellow perch showing positive staining (blue, black arrows) within the glomeruli (scale bar = 50 μm). (b) Tissue section of muscle exhibiting positive stain in endothelial cells (blue, black arrows) (scale bar = 50 μm).

skeletal muscle, liver, pancreas, and adrenal cortex [23]. Given the clinical signs of most infected fish, massive hemorrhages were not as commonly noted as compared to petechia in scattered foci. Changes observed in the kidney and liver were consistent with those from epizootic specimens.

The histopathologic alterations predominantly consist of necrosis and degeneration [1]. Although various tissues are involved, Wolf [1] describes the kidney and liver as the primary targeted organs. Hyperplasia within renal tissue present as foci of macrophages while destruction of melanomacrophage centers can be observed in more advanced stages of disease. Alterations of liver tissue are most notably appreciated in hepatocytes, which often become vacuolated, pyknotic and karyolitic. Aggregations of erythrocytes are also noted within skeletal muscle with minimal damage to fibers or bundles. These findings were initially described in rainbow trout, but were eventually noted in other species including those from the marine environment.

Widespread hemorrhagic changes are the direct result of VHSV's predilection for endothelial cells [49,64,100,101]. As a result, extravasations of the blood supply into the interstitium of muscle and organs are believed to be responsible for the dramatic decrease in circulating blood volume. However, VHSV has also exhibited leukotropic tendencies, especially for macrophages [63,64,68]. Immunohistochemisty demonstrated the presence of VHSV within macrophages and melanomacrophages [64,65], but would be expected as it has a preference for leucopoietic tissues.

Experimental infections have allowed investigators to observe histopathologic changes under controlled conditions. In experimentally infected sea bass (*Dicentrarchus labrax*) and turbot, tissue alterations were observed in kidney tissues which displayed severe interstitial necrosis, while severe necrosis was noted throughout the spleen and liver [80]. When VHSV was initially isolated from whitefish in 1984 [81], the same virus was used for a bath infection of whitefish (*Coregonus* sp.) and rainbow trout. Noted histopathologic changes included focal necrosis of the liver, spleen and pancreas. Additionally, intestinal mucosa was necrotic and the anterior kidney exhibited focal necrosis and degeneration of individual cells with a loss of detail in capillaries. Tubular epithelia were often detached with pyknotic nuclei within a granular cytoplasm. Hyaline casts and necrotic debris were noted in the lumen of tubules while the mesangium of glomeruli were mildly swollen. These findings were also confirmed in histopathologic data from grayling (*Thymallus thymallus* L.) [102]. Similar findings were described in important Great Lakes fish species experimentally infected with VHSV [61] (Fig. 3c and d).

More recently, *in situ* hybridization was performed on histologic sections of yellow perch tissues collected from moribund individuals during an experimental infection using VHSV. The virus was detected in the glomeruli and tubular epithelia of kidney tissues and endothelial cells of muscle (Kim and Faisal, unpublished; Fig. 4). The identification of virus in these particular cells suggests that they are both targets for viral attachment and viral replication. The stage of disease however could not be determined as the tissues were from a moribund fish. As a result, further investigation is warranted whereby a number of tissues collected at varying timepoints following infection could be monitored.

Diagnosis

The diagnosis of VHSV can be accomplished through a number of diagnostic methods. Detailed recommendations by which VHSV can be detected and confirmed are provided by the World Organization for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals 2009 [103] and American Fisheries Society (AFS) Fish Health Section – Bluebook 2007 [104]. A presumptive diagnosis can be completed on the basis of clinical signs, histopathologic changes and through the use of established immortal fish cells lines for initial isolation. Recommended cell lines for freshwater European VHSV isolates have included bluegill fry (BF-2) or rainbow trout gonad (RTG-2) cells in Europe [103–105]. Whereas VHSV isolates from North America can be cultured on *Epitheliosum papulosum cyprini* (EPC), fathead minnow (FHM), BF-2 cells, and chinook salmon embryo (CHSE-214) cell lines [103–105]. Early reports recommend a pH of 7.4–7.8 as ideal for culture medium [106,107], while a pH of 7.1 and temperature of 15 °C in cell culture medium are recommended [108]. Enhancement of viral detection has also been suggested through pretreatment of polyethylene glycol or DEAE dextran [106,109]. Other methods include electron microscopy or gross, clinical, and microscopic pathology. While all of the aforementioned methods are useful in guiding the decisions of the diagnostician, they do not provide a definitive diagnosis for VHSV or any other fish virus given the similarities in clinical signs.

According to the OIE and AFS Bluebook [88,89], confirmation of VHSV can be completed through a variety of serologic techniques. These include the plaque neutralization test (PNT), immunoblot [110,111], fluorescent antibody test [112], enzyme linked immunosorbent assay (ELISA) [111,114–118], and sequence analysis [119]. The PNT technique is dependent on heat-sensitive serum factors known as complement which aid the cross-linking between antigens and antibodies. Serology is also useful to detect viral antigen through the use of indirect fluorescent antibody and immunoperoxidase staining [116,120].

An immunofluorescent technique developed by Jørgensen and Meyling [19] was utilized to identify viral antigens in infected tissues. This technique permits investigators to detect VHSV in tissue during the climax of protein synthesis and assembly, which occurs 18–26 h following infection. The idea is to allow a primary monoclonal antibody to attach to the virus within the tissue, followed by the addition of a secondary antibody that is labeled with a fluorescein isothiocyanate. The same technique was later refined by the same authors in 1974 [121].

As mentioned, ELISA is considered to be the mainstay method by which virus can be detected in cell culture supernatants [111,118]. The method is performed by placing a primary layer, also known as the catching layer, of a polyclonal or monoclonal antibody. Secondly, a blocking reagent, generally bovine serum albumin is then applied to eliminate any extraneous sites that may interfere with the assay. Next the virus is added and then followed by an MAb that will attach to the G protein. The final step is to then to add a tagged anti-species antibody to attach to the last monoclonal antibody added. These methods have been described by Way and Dixon [118] using polyclonal antibodies, Mourton et al. [122] in comparison of indirect, direct and antigen-capture ELISAs.

Virus gene probes have also been developed by Batts et al. [123], which are used to detect complementary deoxyribonucleic acid (cDNA) or viral ribonucleic acids. After thorough investigation, the authors were able to utilize the developed probes in confirmation and differentiation of the two main groups of VHSV. One of the most specific techniques that also has the ability to differentiate serologically similar strains is the reverse transcriptase polymerase chain reaction (RT-PCR). The goal in this case is to obtain the viral RNA and produce a cDNA through the use of a reverse transcriptase and nucleotides within appropriate buffers. The cDNA is then amplified by polymerase chain reaction (PCR) using a TaqPolymerase in a temperature cycler. The amplification of cDNA results in an adequate sample to be electrophoresed in an agarose gel and visualized. This highly specific technique has allowed investigators to identify VHSV more rapidly than other techniques [124–126]. Since most of the recommended primer sets by the OIE and AFS Bluebook can detect all genotypes, it is also recommended that new isolations be sent to a reference laboratory for serologic and molecular analysis.

Prevention and control

At present, the best approach to avoid threats of VHSV infection is by prevention as there are no current chemotherapeutic options available. The primary measure by which fisheries and hatchery managers can prevent the spread of disease is by properly disinfecting contaminated equipment and holding areas and ultraviolet (280–200 nm wavelength) irradiation of incoming water [127]. Other effective control measures include increasing the temperature to above 20 °C [128] or dilution in seawater [129]. However, a recent study investigating the stability of virus in fresh- and saltwater revealed that VHSV, regardless of its environmental origin, could remain stable in either water type [96]. Interestingly the virus survived an average of 13 days in freshwater at 15 °C, yet was inactivated on average of 4 days in seawater. If the temperature of the water was increased in either water type, the stability of the virus was greatly reduced owing to the sensitivity of the virus to higher temperatures. Some disinfectants have also been effective, such as chlorine, hypochlorite, formalin, sodium hydroxide and iodophors [1,49]. Application of any of the aforementioned preventative measures could limit the exposure and recurrence of future outbreaks. Currently, viral hemorrhagic septicemia remains a reportable disease to the World Organization for Animal Health (OIE) which is a critical step in preventing and controlling the spread of the disease before the virus is unintentionally imported and exported through fish and/or fish products. The OIE has also implemented for identifying and confirming the disease which has allowed various countries to initiate management and regulatory guidelines rapidly once the virus was detected.

Vaccination

Vaccine development has shown some promising data in the literature for rainbow trout culture. In 1995, de Kinkelin et al. [130] evaluated vaccination protocols which revealed varying degrees of efficacy and protection between inactivated, live attenuated and recombinant vaccines. The investigation revealed that the virus strain 07/71 inactivated by 2-propiolactone and intraperitoneally injected was effective and immunogenic in trout of all sizes but not all life stages [131]. Cross protection between the three serotypes was observed as well as neutralizing antibodies in the sera of vaccinated fish. The production of live vaccines against VHSV was initiated by Vestergård–Jørgensen by attenuating the virus through several subcultures in rainbow trout gonad cells (RTG-2) at 14 °C. Rainbow trout fry were shown to be protected by immersion up to 150 days following infection at 10 °C. An EPC cell attenuated virus [132] increased the survival rate of 30% in rainbow trout fry when compared to unvaccinated fry. Interestingly, the virus provided immunity against the parent (i.e. pre-attenuated) virus strain but not the wild-type virus.

Subunit vaccines or recombinant virus have also been investigated to determine whether glycoprotein like particles could provide immunity. Given that the glycoprotein gene plays a critical role in antigenicity and virus attachment, studies have revealed that injection of synthetically produced glycoprotein stimulates production of VHSV specific antibodies in rainbow trout [133]. Even more interesting was the production of a subunit vaccine in insect cells through the use of a baculovirus vector [134]. By intraperitoneal injection alone, investigators were able to induce virus neutralizing antibodies and protection against infection in rainbow trout using the baculovirus. These findings were encouraging with respect to vaccination, but the inability to induce protection through immersion made the technique unfeasible for large scale production purposes.

Within the last decade, vaccination against VHSV using DNA plasmid constructs has gained more attention. The earliest studies were those of Lorenzen et al. [135], which

demonstrated that intramuscular vaccination using DNA encoding the glycoprotein gene could induce protective immunity and thus produce neutralizing antibodies in rainbow trout. Alternatively, the nucleoprotein gene was protective but did not induce production of neutralizing antibodies. In 2001, Fernandez Alonso et al. [136] utilized short pulses of low intensity ultrasound to deliver a VHSV DNA vaccine to fingerling rainbow trout. When ultrasound vaccination was compared to immersion or injection, ultrasound delivery of the vaccine was the only method by which humoral immunity and survival post reinfection occurred. Perhaps one of the most promising studies has been through dual DNA vaccination using the glycoprotein gene of infectious hematopoietic necrosis virus (IHNV) and VHSV [137]. After a single intramuscular injection, fish were protected against both viruses for an extended period of time. When DNA vaccination with the glycoprotein gene was conducted on fish acclimated to temperatures of 5, 10, and 15 °C [138], protection against reinfection was conferred at all temperatures. However, non-specific protection, seroconversion and the inflammatory response at the site of vaccination were most rapid at 15 °C. While these studies do not comprise all of the available information on DNA vaccination against VHSV, it does provide compelling and encouraging data.

These early studies eventually sparked numerous trials and investigations into creating an economically feasible, efficacious and practical vaccine. An oral vaccine was recently developed by using a lyophilized virus encased in polyethylene glycol and extruded to create a feed sized pellet [139]. Results indicated that VHSV antibodies could be detected following oral vaccination. After vaccination, the fish were immersion challenged using VHSV strain Fi13 and protection was found to occur, thus presenting a method that could be both effective and feasible in aquaculture settings. Orally vaccinated fish showed significantly lower mortalities compared to fish given a placebo.

In spite of the growing interest in vaccination against VHSV, de Kinkelin [131] indicates that a vaccine must meet several criteria. The vaccine must allow producers to administer the vaccine once without a booster, deliver it easily, purchase it at a reasonable cost and provide universal protection and resistance to infection. Furthermore, the vaccine must not impose the risk of reverting to a virulent form, remain stable in storage and allow investigators to continue sero-surveillance operations. Fulfilling these criteria would be essential in marketing a vaccine that would prevent future economic losses from mass mortalities.

VHSV typing by serological methods

Since the early discovery and isolation of VHSV, scientists realized that VHSV is very heterogenous in nature. For example, based on plaque neutralization assay (PNT) results, three VHSV serotypes have been recognized; Type 1 is primarily represented by the F1 strain (originally isolated from Denmark); Type 2, is represented by the Hededam isolate from Danish rainbow trout [19,77], and Type 3 is represented by the French strain 23/75, isolated from brown trout by de Kinkelin and Le Berre [20]. The experiments designed by Olesen et al. [140] were critical in providing a more detailed characterization of VHSV serogroups. The author was able to demonstrate that the three serogroups while different, were in actuality descendents of one another, explaining the subtle differences and overlap between serogroups. Such discrepancies encouraged investigators to pursue alternative methods of differentiating isolates through sequence comparisons of the glycoprotein gene for phylogenetic analysis.

Phylogeny

Once believed to be a disease unique to cultured rainbow trout, subsequent reports demonstrate that VHSV has both a wide host and geographic range. The compiled lists of isolations from various fish species and their locations throughout the last two decades have been instrumental in unraveling the phylogenetic relationships that exist between isolates. Among the earliest phylogenetic studies on VHSV were those performed by Benmansour et al. [141] and Stone et al. [47], who sequenced the G-gene from a number of VHSV isolates and reported the presence of three distinct genotypes, which correlates with geographic ranges. Genotype I included isolates from Europe, Genotype II included isolates from in and around the British Isles, and Genotype III included North American isolates. Prior to that the distinction between North American and European isolates was suggested by Oshima et al. [142], Bernard et al. [143], and Batts et al. [123]. The studies of Benmansour et al. [141] and Stone et al. [47] ignited more interest in phylogenetic studies on VHSV as opposed to continue the serologic testing for strain differentiation. This approach was so sensitive that it differentiated among various isolates within the same genotype. For example, Thiéry et al. [144], who studied the sequence of the V2 region of the G gene of 63 French VHSV isolates, concluded the presence of four sublineages (designated a–d) within Genotype I.

In 2004, Snow et al. [119] published the most comprehensive phylogenetic tree that has lead to current understanding of VHSV genotypes and their geographic ranges. Using 128 VHSV isolates from numerous geographic and host origins, the authors were able to validate previously reported molecular epidemiological data and gain insights into the subtle differences within each genotype. Analysis of the isolates revealed that Genotype I included freshwater farmed rainbow trout in continental Europe, denoted Ia, [47,104,141] and marine isolates from the Baltic Sea, Skagerrak and Kattegat, denoted Ib [104]. Interestingly Genotype Ia included one marine isolation from farmed turbot in Germany, which was surprising given that all of the other isolates of the sublineage were from freshwater. Some studies have also included additional sublineages of Genotype I to include Ic, Id, and Ie [145], all of which are rainbow trout. The close designation of this marine isolation to outbreaks in freshwater, was previously suggested by Schlotfeldt et al. [31] and attributed to virus contaminated effluent from farms containing infected rainbow trout. Isolates of Genotype Ib also contained one isolate from Atlantic herring from the English Channel [37], which suggests the migratory behavior of herring may have been responsible for this unlikely inclusion. While such differences were critical in understanding VHSV's ability to expand in new locales, the most significant was the close genetic relationship between isolates of Genotype Ia (freshwater) and Ib (marine), which, combined with the findings of Einer Jensen et al. [145], strongly suggested that the origin of VHSV in outbreaks of rainbow

Table 1 Phylogenetically classified viral hemorrhagic septicemia virus isolates by species and location.

Genotype	Sub-group	Species	Location	Source
I	Ia	Rainbow trout, *Oncorhynchus mykiss*	France, United Kingdom	[47,119]
	Ic		Denmark	[145]
	Id		Vestrefjord, Norway	[145]
	Ie		Georgia	[145]
	Ia	Turbot, *Scophthalmus maximus*	Germany	[31]
	Ie		Black Sea	[151]
	Ib	Atlantic herring, *Clupea harengus*	Baltic Sea	[38]
		Sprat, *Sprattus sprattus*	Baltic Sea	[91,152]
		Dab, *Limanda limanda*	Kattegat	[145]
		Sand goby, *Pomatoschistus minutus*	Baltic Sea	[119]
		Fourbeard rockling, *Enchelyopus cimbrius*	Baltic Sea	[38]
		Blue whiting, *Micromesistius poutassou*	North Sea	[91,146,147]
		Japanese flounder, *Paralichthys olivaceus*	Japan	[147]
		Sprat, *Sprattus sprattus*	Baltic Sea	[38]
II	II	Atlantic Cod, *G. morhua*	Baltic Sea	[38]
		Atlantic herring, *Clupea harengus*	Baltic Sea	[38]
		Whiting, *Merlangius merlangus*	North Sea	[38]
III	III	Turbot, *Scophthalmus maximus*	Ireland	[146]
		Norway pout, *Trisopterus esmarkii*	North Sea	[152]
		Haddock, *Melanogrammus aeglefinus*	North Sea	[153]
		Atlantic Cod, *G.morhua*	North Sea	[153]
		Atlantic herring, *Clupea harengus*	Skagerrak	[38]
		Argentine, *Argentina sphyraena*	North Sea	[38]
		Eel, *Anguilla anguilla*	France	[12,154,155]
		Greenland halibut, *Reinhardtius hippoglossoides*	Flemish Cap	[156]
		Rainbow trout, *Oncorhynchus mykiss*	Norway	[48]
		Japanese flounder, *Paralichthys olivaceus*	Japan	[41,157,158]
IV	IVa	Atlantic salmon, *Salmo salar*	Canada, Pacific	[159,160]
		Pacific herring, *C. pallasii*	British Columbia (BC)/Alaska/ Washington (WA), USA	[141]
		Pacific sardine, *Sardinops sagax*	BC, Oregon, California	[34]
		Pacific cod, *G. macrocephalus*	Prince William Sound, Alaska, USA	[141]
		Chinook salmon, *O. tshawytscha*	Orcas Island, WA, USA	[24]
		Coho salmon, *O. kisutch*	Makah, WA, USA	[141]
		Pacific sardine, *Sardinops sagax*	BC, Oregon, California	[34]
		Pacific mackerel, *Scomber japonicus*	Santa Catalina, CA, USA	[34]
		Eulachon, *Thaleichthys pacificus*	Sandy River, OR, USA	[34]
		Shiner perch, *Cymatogaster aggregata*	NW Vancouver Island, British Columbia (BC), Canada	[34]
		Surf smelt, *Hypomesus pretiosus*	Winchesterbay, OR, USA	[34]
		Sablefish, *Anoplopoma fimbria*	Queen Charlotte Straits, BC, Canada	[34]
		Muskellunge, *Esox masquinongy*	Lake St. Clair, MI, USA	[43]
	IVb	Brown trout, *Salmo trutta*	French River, Nova Scotia	[46]
		Striped bass, *Morone saxatilis*	Miramichi Bay and River, Baie du Vin, New Brunswick	[46]
		Mummichog, *Fundulus heteroclitus*	Ruisseau George Collette, near Bouctouche, New Brunswick	[46]
		Three-spined stickleback, *Gasterosteus aculeatus aculeatus*	Ruisseau George Collette, near Bouctouche, New Brunswick	[46]
		Freshwater drum, *Aplodinotus grunniens*	Lake Ontario, Canada	[44]
		Round gobies, *Neogobius melanostomus*	Lake Ontario and St. Lawrence River, NY, USA	[45]

trout was from the marine environment. Genotype II included 7 isolates of which were from the Baltic Sea, none of which were linked to rainbow trout aquaculture. This second marine lineage thus provided even more evidence that VHSV originated in the marine environment. Genotype III consisted of over 30 isolates from the British Isles and 2 isolates from Irish and Scottish turbot marine farms. Lastly, Genotype IV included marine isolates from North America and Japan.

The discovery and classification of Genotype IV isolates from coho and chinook salmon in Washington State [42,146–148] reinforced the idea that VHSV was from the marine environment. Surprisingly, in 2003, VHSV was isolated (MI03) from muskellunge caught from the freshwater Lake St. Clair in Michigan [43]. Sequence analysis of G and N genes of MI03 revealed that the Great Lakes isolate was closely related to the North American strain, yet slightly distinct. The authors suggested the establishment of a new sublineage within Genotype IV, designated as IVb with MI03 as the index strain while the Pacific Northwest strains constituted the IVa sublineage within Genotype IV with the Makah strain as the index strain. Subsequent isolations from a number of fish species within the Great Lakes basin and the east Atlantic coast revealed the widespread distribution of VHSV-IVb and its wide host range [44–46]. Sequence data from the virus recovered from these hosts revealed a nearly identical match to the original MI03 muskellunge isolate, which suggested an even more extensive host range than any other isolate of VHSV. Table 1 displays the current status of phylogenetic analysis of VHSV isolates retrieved from all over the world.

The conservative nature of the N-gene has proven useful in prolonged evolutionary comparisons of other *Lyssavirus* spp. [149,150] and speculating the origins of new isolates. Partial sequences of the G and P genes revealed that the Japanese isolates belong to two genotypes; seven were closely related to North American isolates, while only one isolate was more closely related to European isolates. In the same context, an overwhelming influx of newly described isolates suggested that VHSV originated from the marine environment. Einer Jensen et al. [145], who compared the entire G-gene sequences from 74 isolates retrieved from freshwater and marine fish worldwide, demonstrated that the North American and European isolates' division occurred approximately 500 years ago, while the ancestor of the European freshwater isolates emerged relatively recently (~50 years prior). The authors noticed that freshwater isolates have a 2–5 times faster nucleotide substitution rate, which they interpreted as either a result of inherent physiologic differences from fish hosts (marine vs. freshwater), or a consequence of intense aquaculture practices. Through restriction fragment length polymorphism assays, Einer Jensen et al. [148] determined that Japanese isolates were closely related to Genotype IV with the exception of one, which was from Genotype I. Although the discovery of Genotype IV isolates in Japan are not surprising, the identification of a Genotype I isolate suggests the possibility of accidental importation of infected fish from a foreign country. Consequently, the use of phylogenetic data remains useful in understanding the patterns of distribution between different genotypes and will continue to be a critical tool in understanding the epizootiology of VHSV.

More recently VHSV was isolated from rainbow trout in the United Kingdom [47], which demonstrated the first VHSV isolate from the United Kingdom (UK) obtained from a freshwater species in a freshwater environment. Additionally, previous isolations in the region were more closely related to Genotypes III and Ib [145], whereas the newest UK isolate was considered to be of Genotype Ia based on G-gene sequences. The re-emergence of VHSV was also experienced in Norway in seawater-farmed rainbow trout [48]. Investigators determined the isolate was from Genotype III, which has not been isolated from salmonids. The authors concluded that the outbreak was most likely a result of viral transmission from the marine environment. Both isolations have thus confirmed VHSV's capacity to engage new hosts and challenge the notion that genotype and geography were strongly related. The number of species found within a particular genotype is described in Table 1.

Knowledge gaps

In general, our current understanding of VHSV has been largely provided by published data regarding European and Pacific VHSV isolates. Despite the obvious similarities in disease manifestations, there is clear evidence that not all VHSV isolates carry the same host-range, function at equivalent environmental parameters, or contain identical genomes. Such differences have compelled investigators to characterize the virus and assess the risk to neighboring species, while also providing regulatory agencies the ability to make informed decisions.

At present, little data have been made available regarding the pathogenicity and host susceptibilities of recent VHSV isolates. Furthermore, little is known about the degree of pathogenicity of VHSV for listed and unlisted freshwater and marine species from which VHSV has been isolated from, nor is there a clear distinction between species that are resistant, become reservoirs, or develop fulminant VHSV infection. While such data would be critical in guiding policy and management, the occurrence of VHSV infections in previously unidentified species each year has owed to VHSV's unpredictable host range. This in combination with the devastative nature of VHSV has sparked the dilemma for fisheries and hatchery managers from a recreational and economic standpoint.

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