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Complete sequences of 4 viral hemorrhagic septicemia virus IVb isolates and their virulence in northern pike fry

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ABSTRACT: Four viral hemorrhagic septicemia virus (VHSV) genotype IVb isolates were sequenced, their genetic variation explored, and comparative virulence assayed with experimental infections of northern pike *Esox lucius* fry. In addition to the type strain MI03, the complete 11 183 bp genome of the first round goby *Neogobius melanostomus* isolate from the St. Lawrence River, and the 2013 and 2014 isolates from gizzard shad *Dorosoma cepedianum* die-offs in Irondequoit Bay, Lake Ontario and Dunkirk Harbor, Lake Erie were all deep sequenced on an Illumina platform. Mutations documented in the 11 yr since the MI03 index case from Lake St. Clair muskellunge *Esox masquinongy* showed 87 polymorphisms among the 4 isolates. Twenty-six mutations were non-synonymous and located at 18 different positions within the matrix protein, glycoprotein, non-virion protein, and RNA polymerase genes. The same 4 isolates were used to infect northern pike fry by a single 1 h bath exposure. Cumulative percent mortality varied from 42.5 to 62.5%. VHSV was detected in 57% (41/72) of the survivors at the end of the 21-d trial, suggesting that the virus was not rapidly cleared. Lesions were observed in many of the moribund and dead northern pike, such as hemorrhaging in the skin and fins, as well as hydrocephalus. Mean viral load measured from the trunk and visceral tissues of MI03-infected pike was significantly higher than the quantities detected in fish infected with the most recent isolates of genotype IVb, but there were no differences in cumulative mortality observed.

KEY WORDS: *Esox lucius* · Experimental infection · Illumina sequencing · Northern pike · Viral hemorrhagic septicemia virus · VHSV IVb

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV, family *Rhabdoviridae*, genus *Novirhabdovirus*, species *Piscine novirhabdovirus*) causes a serious disease of fishes in both wild and cultured environments (Wolf 1988). The geographic distribution of VHSV extends throughout the Northern Hemisphere and it has been isolated from dozens of marine and freshwater fish species (Faisal et al. 2012, OIE 2016). VHSV is grouped into 4 major genotypes, each from a distinct geographic region of the world (Skall et al. 2005).

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Genotype I include isolates from Europe and is divided into 5 sublineages (a–e); genotype II has been found in the Baltic Sea; and genotype III strains are from the North Sea, Skagerrak, and Kattegat areas of northern Europe (Stone et al. 1997, Snow et al. 1999, 2004, Einer-Jensen et al. 2004, 2005, Elsayed et al. 2006, Dale et al. 2009). Genotype IV comprises isolates from the United States and Canada in North America, and Japan, Korea, and China in Asia (Meyers & Winton 1995, Takano et al. 2000, Nishizawa et al. 2002, Hedrick et al. 2003, Kim et al. 2003, Einer-Jensen et al. 2005, Elsayed et al. 2006, Kim et al. 2009, Zhu & Zhang 2014). VHSV infections have caused severe mortalities at olive flounder *Paralichthys olivaceus* farms in Korea and Japan (Isshiki et al. 2001, Kim et al. 2009) and isolations of genotype IVa from several marine fish species on both Korean and Japanese coasts have been reported (Takano et al. 2000, Watanabe et al. 2002, Kim et al. 2011). Molecular characterization of North American and Asian VHSV isolates has identified at least 2 sublineages (Elsayed et al. 2006, Smail & Snow 2011): genotype IVa from the Northern Pacific Ocean, including western North America and Asia (Meyers & Winton 1995, Nishizawa et al. 2002, Hedrick et al. 2003, Einer-Jensen et al. 2005, Kim et al. 2011) and genotype IVb from the freshwaters of the North American Laurentian Great Lakes (Gagné et al. 2007, Groocock et al. 2007, Lumsden et al. 2007, Thompson et al. 2011). An additional subgroup (IVc), consisting of 4 marine isolates from the Atlantic coast of North America, has been proposed (Pierce & Stepien 2012).

The first isolation of VHSV IVb came from adult muskellunge *Esox masquinongy* captured in Lake St. Clair, Michigan in 2003, generating the index type strain MI03 (Elsayed et al. 2006). In 2005, VHSV emerged in the Great Lakes as the cause of large-scale die-offs in several fish species, and by 2009 VHSV was detected throughout the entire Great Lakes basin (Bain et al. 2010, Hope et al. 2010, Cornwell et al. 2011, Frattini et al. 2011, Thompson et al. 2011, Faisal et al. 2012). Since 2005, several laboratories have conducted extensive disease diagnostic investigations, surveillance efforts, and transmission trials with the MI03 type strain of VHSV genotype IVb to determine the relative susceptibility of various Great Lakes fish species to infection and disease (Elsayed et al. 2006, Kim & Faisal 2010a,b, Groocock et al. 2012, Cornwell et al. 2013a,b,c, Getchell et al. 2013, 2015, Grice et al. 2015, Coffee et al. 2017). These studies revealed dramatic variation in susceptibility, and laboratory immersion challenges of juvenile muskellunge with VHSV genotype IVb reported high susceptibility, with a lethal dose that kills 25% of the population (LD_{25}) of 1.4×10^3 plaque-forming units (PFU) ml^{-1} (Kim & Faisal 2012). Northern pike *Esox lucius*, a species closely related to muskellunge, are large keystone piscivores that are important in 'top-down' predatory regulation of the fish community within the Great Lakes ecosystem (Casselman & Lewis 1996). Pike are a common and abundant species, found in 45% of the total freshwater area of North America (Carlander et al. 1978). They have not previously been tested for their relative susceptibility to VHSV IVb. Northern pike were one of the first non-salmonid species infected with the original Egtved strain F1 by European fish virologists (Meier et al. 1994).

To characterize the genetic diversity of Great Lakes VHSV IVb isolates at the early stage of emergence, Thompson et al. (2011) compared a partial glycoprotein (G) gene sequence (the 669 nt vcG region) of 108 isolates collected from 2003 to 2009 from 31 species and 37 sites. This revealed 11 different genotypes designated vcG001–vcG011. Stepien et al. (2015) sequenced and compared the non-virion (NV), phosphoprotein (P), and matrix (M) genes from 10 of these early VHSV genotypes as well as 2 variants isolated from Lake Erie fish in 2012. Several additional vcG genotypes detected in Great Lake fish surveillance samples are currently being characterized (G. Kurath, W. N. Batts unpubl. data).

Since 2009, there have been fewer reported outbreaks of VHSV in the Great Lakes, suggesting that genotype IVb has become endemic and potentially less virulent (Kim & Faisal 2011, Pierce & Stepien 2012, Throckmorton et al. 2015). A reduction in observed disease could also be due to a change in the host populations, such as increased herd immunity relative to the naive populations when the virus first emerged (Wilson-Rothering et al. 2015). A limited number of amino acid changes could alter VHSV virulence (Betts & Stone 2000, Kim et al. 2014, Ito et al. 2016). Emmenegger et al. (2013) explored differences in virulence between isolates from VHSV subtypes Ia, IVa, IVb, and IVc. Alignment of complete genome sequences alone, without parallel infection trials, has not provided a correlation between variations in the viral proteins and the virulence of VHSV isolates (Betts & Stone 2000, Kim et al. 2013, Einer-Jensen et al. 2014, Ito et al. 2016). Our goal was to continue the search for genetic traits determining VHSV virulence mechanisms and characterize the genome-wide genetic diversity of Great Lakes IVb VHSV. The present effort compares the virulence of 4 VHSV IVb isolates using experimental infections of

northern pike fry in addition to sequencing the complete genome of each of the 4 isolates and assessing the genetic changes in 3 more recent VHSV variants from New York compared with the index strain MI03.

MATERIALS AND METHODS

Fish

Northern pike *Esox lucius* fry spawned from native brood fish captured in the St. Lawrence River near Clayton, NY were raised for 2 mo at the Thousand Island Biological Station on well water. On 20 June 2016, approximately 200 of these fry were transported back to the Aquatic Animal Health Program facility at the Cornell University College of Veterinary Medicine. All fish were initially held in a 700 l fiberglass tank (Frigid Units) at 15 ± 1°C with flow-through, chlorine-free, filtered water. After a brief 2-d confinement, 160 northern pike were randomly placed into a series of sixteen 37 l tanks in a flow-through system (10 fish tank^{-1}). The randomization list was generated online at random.org (Haahr 2011). Another 40 fish of the same population were randomized into 4 similar tanks in a separate room and designated as the controls. The water temperature was then lowered each day by 1.0°C until the temperature was 13 ± 1°C, an optimal temperature for VHSV infection (Wolf 1988). Fish were inspected daily, maintained on a photoperiod of 12 h of daylight, and fed cultured brine shrimp. Throughout the experiments, strict biosecurity protocols were maintained to ensure virus was not transferred between tanks of fish or outside of the experimental room. Wastewater from the exposed tanks was disinfected with 12.5% sodium hypochlorite to maintain a free residual chlorine concentration of at least 1.0 mg l^{-1} for at least 10 min before discharge into the municipal sewage lines. The Cornell University Institutional Animal Care and Use Committee approved all use of animals.

VHSV challenge isolates

The challenge isolates consisted of the MI03 isolate of VHSV from muskellunge (genotype vcG001), the first round goby *Neogobius melanostomus* isolate from the St. Lawrence River (vcG002, GenBank accession no. EF564588, Groocock et al. 2007) and the 2013 and 2014 isolates from gizzard shad *Dorosoma cepedianum* die-offs in Irondequoit Bay, Lake Ontario (vcG060) and Dunkirk Harbor, Lake Erie (vcG015, Lewis et al. 2017). These isolates will be referred to hereafter by their vcG genotypes. VHSV IVb isolates vcG002, vcG015, and vcG060 were originally cultured from combined aliquots of spleen, heart, liver, and anterior and posterior kidney from each fish, which were pooled in groups of 3 or 4 (Table 1). VHSV IVb isolate vcG001 (MI03) was kindly provided by Dr. Mohamed Faisal, Michigan State University. The vcG designations were derived from partial glycoprotein (G) gene sequences (Thompson et al. 2011). Isolates were propagated in *epithelioma papulosum cyprini* (EPC, Winton et al. 2010) cells, harvested when the average cytopathic effect was at least 80%, stored in HMEM-10 media at −80°C (Groocock et al. 2007), and quantified using standard plaque assay protocols (Batts & Winton 1989) and RT-qPCR as described below. The viral stocks were not plaque purified and a low passage number was utilized to keep the isolate's heterogeneity that would be found in the field.

Immersion trial

Experimental infection of northern pike fry by immersion

Each set of four 37 l tanks was randomly assigned a viral isolate and 2 of these tanks randomly assigned to receive either a high dose (5×10^5 PFU ml^{-1}) or a low dose (5×10^4 PFU ml^{-1}) of 1 of 4 strains of VHSV

Table 1. Viral hemorrhagic septicemia virus (VHSV, Genotype IVb) isolates used in this study. Viral sequence types are indicated by a universal sequence designator (USD) based on the 669 nt partial glycoprotein gene sequence (vcG) used previously for genetic typing of Great Lakes VHSV (Thompson et al. 2011). Medium from cell cultures showing cytopathic effect (CPE) was stored in aliquots at −80°C for use as stock virus. EPC: *epithelioma papulosum cyprini*

Isolate	USD	Host	Geographic origin	Year isolated	Passage number
MI03	vcG001	Muskellunge	Lake St. Clair, MI	2003	3rd pass EPC
FPL2006-005	vcG002	Round goby	St Lawrence River, NY	2006	2nd pass EPC
FPL2013-002	vcG060	Gizzard shad	Irondequoit Bay, Lake Ontario, NY	2013	2nd pass EPC
FPL2014-001	vcG015	Gizzard shad	Dunkirk Harbor, Lake Erie, NY	2014	2nd pass EPC

IVb (vcG001, vcG002, vcG015, or vcG060) for a total of 8 treatments in the 16 tanks. Fish were immersed in groups of 10 fish l^{-1} of static, VHSV-containing water for 1 h, and subsequently returned to the flow-through system tanks. The 40 control fish were also immersed in groups of 10 fish l^{-1} of static, HMEM-containing water for 1 h, and subsequently returned to the flow-through system tanks. The fish were monitored twice per day and their mortalities recorded daily for 21 d. Moribund fish were counted as mortalities, removed from their tanks, euthanized with an overdose of tricaine methanesulfonate (MS-222, Western Chemical) buffered 1:1 with sodium bicarbonate, and external signs of infection such as petechial hemorrhages of the skin, fin, and eye were recorded. The fish were then severed anterior to the cardiac and abdominal cavities to separate the head and trunk, which were placed directly into different 2 ml homogenizing tubes containing 200 µl RNAlater (Ambion) and stored at −80°C. A single moribund day-21 fish with signs of hydrocephalus was examined by optical coherence tomography and then fixed in 10% neutral buffered formalin (v/v) for 48 h and then placed in a decalcification solution for at least 1 wk prior to histological evaluation. A day-21 fish was also randomly selected from a control tank, euthanized, and decalcified. These 2 fish were routinely processed, sectioned at 5 µm, and stained with hematoxylin and eosin. The forceps and scalpels were disinfected with 20% household bleach between each specimen (3 to 6% sodium hypochlorite, final solution approximately 10 000 mg l^{-1} active chlorine; contact time at least 30 s). At the end of the trial, surviving fish also were checked for external signs of infection and the total length (mm) and weight (g) of 10 control fish were recorded. The mean length was 30.9 ± 2.3 mm and the mean weight was 0.23 ± 0.041 g. Each of the survivors was euthanized and processed as described above.

RNA extraction and RT-qPCR testing

Sample extraction and molecular testing procedures closely followed those used by Getchell et al. (2015). A brief summary includes the addition of 250 µl of HMEM-10 and a single steel bead to each sample prior to homogenization with a Minibeater-16 (BioSpec Products); centrifugation at 3000 × *g* for 2 min; and extraction of 50 µl of each supernatant with a MagMax-96 viral RNA isolation kit (Life Technologies). The primer and probe sequences utilized in the RT-qPCR assay have been described by Hope et al. (2010). RT-qPCR efficiency between isolates was very similar (data not shown). RNA quality assessment was measured with a Q3000 spectrophotometer (Quawell Technology). Viral copy number results were expressed as genome equivalents per 50 ng^{-1} total RNA.

Virus sequencing and genome analyses

RNA extraction and PCR

Prior to submitting these isolates for sequencing, RNA extractions were conducted following the procedures described above with the MagMax-96 viral RNA isolation kit. Next, preparatory PCR was conducted following the procedures published by Schönherz et al. (2016). Briefly, first-strand cDNA was generated with SuperScript III Reverse Transcriptase (Invitrogen). The reverse transcriptase product and DNA quality and concentration were assessed by agarose gel electrophoresis and spectrophotometry. PCR amplification of the complete viral genome was accomplished with 4 primer sets (Schönherz et al. 2016), but with some changes in the cycling conditions. The annealing temperatures were increased by 5°C and the number of cycles decreased to 29 (6 in the touchdown phase, 23 in the fixed annealing temperature of 55°C). Only 1 µl of cDNA was amplified in each 20 µl PCR reaction using New England Biolabs' (NEB) One Taq DNA polymerase. Amplification was performed for all 4 isolates and 4 fragments (2797 to 3709 bp) were generated from each isolate. The PCR products were cleaned with a QIAquick PCR Purification Kit (Qiagen). Subsequently, amplicons of the same isolate were adjusted to equivalent concentrations and combined into one sample.

Viral sequencing and genome assembly

Pooled samples from all 4 isolates were sheared to 300 bp with a Covaris S2 sonicator. Resulting fragments were purified with Ampure XP beads (Beckman Coulter), end polished with T4 polynucleotide kinase and T4 DNA polymerase, adenylated with *Taq* polymerase, and ligated to universal Truseq adapters with T4 DNA ligase (all enzymes from NEB). Each sample was indexed by PCR (15 cycles) with a universal forward primer and a unique Truseq index primer, with One Taq DNA polymerase (NEB). The resulting library was sequenced on an Illumina MiSeq platform

(paired 250 bp reads) at the BioResource Center, Cornell University. High-quality reads trimmed of adapter and primer sequences were aligned to a VHSV IVb whole genome (Ammayappan & Vakharia 2009) in NGen (v.12, DNASTAR). Single nucleotide polymorphisms (SNP) were called from the resulting SeqMan (.assembly) file, from the SNP report menu.

Phylogenetic and selection analyses

The 4 Great Lakes VHSV genome sequences determined here (KY359354-57) were aligned with 24 other full-length VHSV genome sequences available in GenBank, including 12 genotype I, 1 genotype II, 3 genotype III, 7 genotype IVa, and 1 genotype IVb (the MI03 reference strain). The 28 sequences ranged in length from 10 845 to 11 184 nt, and all contained the complete coding regions of the genomes (from start of the nucleoprotein (N) gene open reading frame (ORF) to the end of the RNA polymerase (L) gene ORF), but with variable lengths of the non-coding regions at the 3′ and/or 5′ termini. The 28 genome sequences were aligned using MUSCLE (Edgar 2004). The resulting alignment was used to construct a phylogenetic tree in RAxML (Stamatakis 2006), applying GTR + gamma (4 rate categories). Markov chain Monte Carlo (MCMC) chains (2 runs and 4 chains each) were run for 250 000 generations in Mr. Bayes. Twenty starting trees were generated and support values were generated from 500 bootstrap replicates.

To assess the potential role of selection in the diversification of the IVa genomes, we implemented 2 different codon based methods for each loci: FUBAR (fast, unconstrained Bayesian approximation, Murrell et al. 2013) and MEME (mixed effects model of evolution, Murrell et al. 2012). FUBAR is a maximum likelihood method that allows the rate of non-synonymous (dN) and synonymous (dS) substitutions to vary across codons according to a number of site classes given *a priori*. This allows for testing codons for positive (dN/dS > 1) or purifying (dN/dS < 1) selection. While FUBAR assumes that selection is constant, MEME allows the distribution of dN/dS to vary from branch to branch, making it possible to detect episodic selection. One of the advantages of the MEME approach is its capability of detecting positive selection even under the strong influence of purifying selection. However, FUBAR is considered to be a more robust test since it is less sensitive to model specification, particularly when the strength of selection varies across different sites.

Because intragenic recombination is known to elevate the false positive error for positive selection, we first tested for recombination using the GARD genetic algorithm. GARD, FUBAR, and MEME analyses were run in HyPHY as implemented in the DataMonkey server (www.datamonkey.org). Significance was assessed by posterior probability > 0.9 (FUBAR) and p-values < 0.05 (MEME).

Glycoprotein structure analysis

The amino acid sequences from all 4 glycoproteins were submitted to the Phyre2 web portal (Protein Homology/analogY Recognition Engine V 2.0) for protein modeling, prediction, and analysis (Kelley et al. 2015).

Statistical analysis

Cox proportional hazard model was used to test the significance of the covariates of dose and virus strain on risk of death after exposure to virus and was assessed using the likelihood ratio test for significance (Cox & Oakes 1984). Survival time was defined as the number of days until a fish was sampled due to morbidity or death or at the conclusion of the trial. Testing the proportionality assumption of the Cox model was performed using the 'cox.zph' function. Influential observations were visualized by plotting the deviance residuals using the 'ggcoxdiagnostics' function. Nonlinearity was not an issue for categorical variables, so that assumption did not need to be tested. Viral loads between samples in head and brain tissues and trunk and visceral tissues (run separately) were compared using a 2-way ANOVA. Statistical comparisons were performed using R statistical software through RStudio Version 0.99.903 and considered significant at p-values < 0.05.

RESULTS

Immersion challenge

Of 160 exposed northern pike *Esox lucius*, 77/84 (91.7%) moribund or dead fish, and 41/72 (56.9%) survivors tested positive for VHSV by RT-qPCR, except 4 fish missing at the end of the trial, possibly due to cannibalism. Overall, there was 42.5% cumulative percent mortality in the low-dose groups versus 62.5% cumulative percent mortality in the

high-dose groups. Thirty-five percent (25/72) of the survivors were positive by RT-qPCR of the trunk and visceral organ samples, and 29% (21/72) of survivors were positive by RT-qPCR of the head and brain samples. Viral RNA load ranged from 5.4 million down to 3 N-gene copies per 50 ng of total RNA, and the means of each treatment are shown in Fig. 1. All fish in the study underwent a gross examination. Moribund and dead fish showed signs of hemorrhaging on the caudal and anal fins as well as moderately injected vessels and petechial hemorrhaging on the trunk (Table 2, Fig. 2A–C). Protrusions through the dorsal side of the cranium suggested hydrocephalus (Fig. 2B,D). A single specimen was examined by optical coherence tomography (OCT) and histology (Fig. 3). The OCT scan of this northern pike at day 21 post-infection showed a swelling of the optic tectum. The histological section of the same specimen showed an increased size of the brain compared with the age-matched control fish. Many virus-exposed fish also died with no clinical signs of disease. No internal observations were made, due to their small size and to preserve the integrity of the tissues for RT-qPCR analyses. The presence of different abnormalities was not compared across isolates, but more fish in the high-dose groups showed signs of infection, compared with the low-dose groups (Table 2). The appearance of swelling of the brain between 8 to 16 d post-infection occurred after the hemorrhaging observed earlier in the 21-d trial. The trunk and head samples of some surviving experimental fish also showed high loads (>1 × 10^3 per 50 ng of total RNA) of VHSV in 7 of 41 and 6 of 41 positive fish, respectively. None of the control fish showed any clinical signs of VHSV or tested positive by RT-qPCR when their tissues were assayed, although the deaths of 8 of 40 (20%) fish in the first 4 d was higher than 7 of the virus-exposed groups, but comparable to 1 virus-exposed group with deaths of 6 of 20 (30%) during this early time period (Fig. 4).

Survivorship curve and hazard ratio

Approximately half the experimental fish died (or were moribund and euthanized) between day 1 and day 15 (Fig. 4). A Cox proportional hazards model was fit to the data. There was no significant interaction between treatment (dose) and vcG type, so this interaction was removed from the model. The results of the Cox regression on survival data did demonstrate a difference when comparing the time to death of the high dose versus the low dose over the course of the experiment (likelihood ratio test: df = 2,

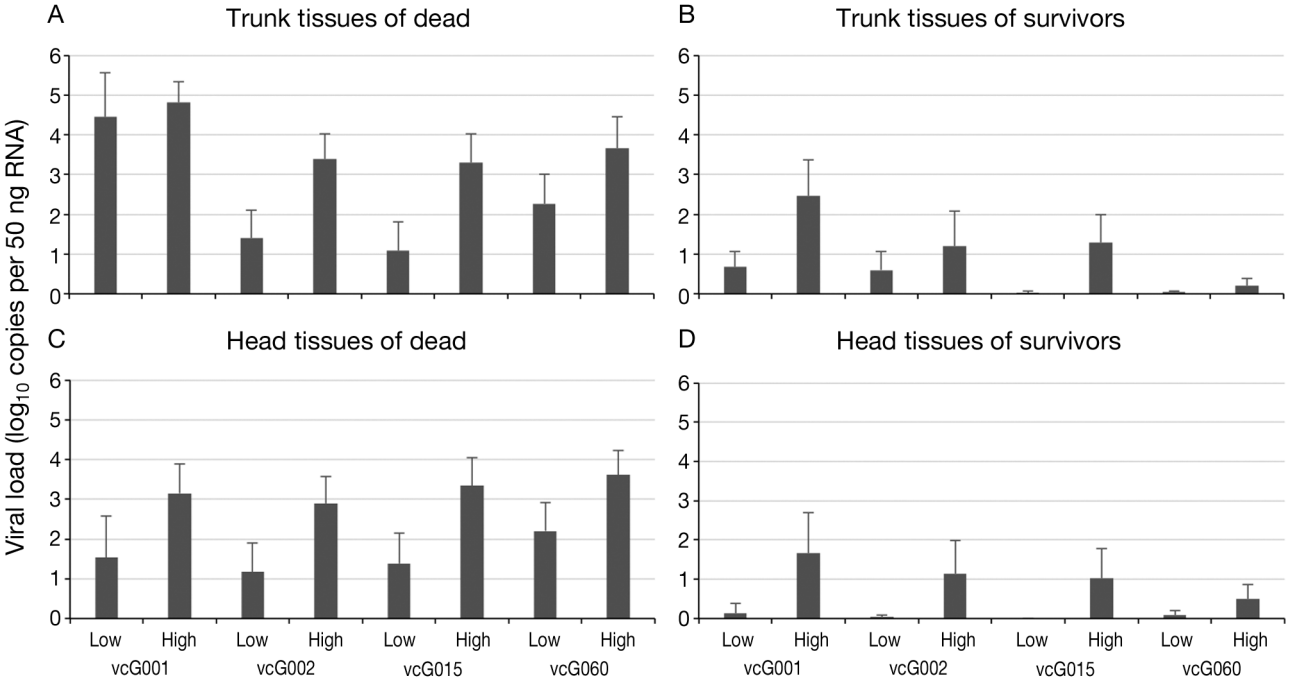

Fig. 1. Average viral load in northern pike *Esox lucius* (A,C) mortalities and (B,D) survivors infected with 4 different strains of viral hemorrhagic septicemia virus IVb at 2 doses: (A,B) trunk and visceral and (C,D) head and brain tissue. The *x*-axis shows the virus strain names and dose level. The *y*-axis shows $\log_{10}$ nucleoprotein gene copies per 50 ng of total RNA. Error bars are SE

Table 2. Signs observed in northern pike during viral hemorrhagic septicemia virus immersion challenge. Fish were monitored on a daily basis, but only those days when clinical signs were noted are listed here. Commas seperate individual fish; plus (+) symbols indicate fish with more than 1 lesion type. A: anal fin hemorrhage; B: brain swelling/hydrocephalus; C: caudal fin hemorrhage; E: eye hemorrhage; H: head hemorrhage; T: trunk hemorrhage

Days post challenge	vcG001 Low	vcG001 High	vcG002 Low	vcG002 High	vcG015 Low	vcG015 High	vcG060 Low	vcG060 High
5	T	T						
6		A+C+E, T				A, T, T		T
7	E+T, T	C+T, T		T		H	H, T	T
8		C+T, B+T, B, T		T, T		T		T, T
9		B, T		T		T		T
10		T	B+T	C+T, T		B		
12			T			B, B		T
13			A+B	B	B			B
14								B
15	B+T			B, B, T				
16							B	
17				H				
21								B
Total	4/20	12/20	3/20	11/20	1/20	9/20	3/20	9/20

χ^2 = 12.88, p = 0.0016). No difference was observed when comparing the time to death of the vcG strains over the course of the experiment (df = 2, p = 0.607). Validity of the proportionality of hazards assumption was confirmed for the model using the 'cox.zph' function. The test was not statistically significant for each of the covariates (dose: χ^2 = 2.440, p = 0.118; vcG: χ^2 = 0.136, p = 0.721), and the global test was also not statistically significant (χ^2 = 2.615, p = 0.270). The deviance residuals were roughly symmetrically

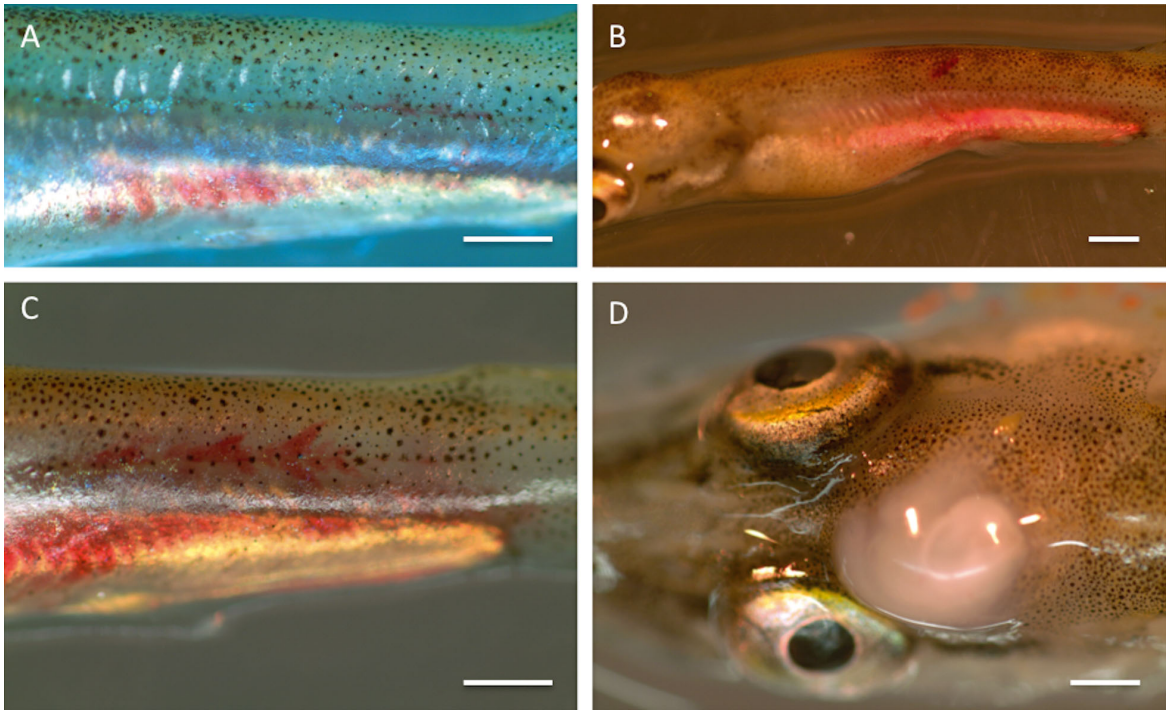

Fig. 2. Pathology associated with viral hemorrhagic septicemia virus in northern pike *Esox lucius* fry experimentally exposed to genotype IVb isolates by immersion challenge. (A) Day 8 post-infection with hemorrhages along lateral line and within myomeres, vcG001 high dose (scale bar = 2 mm). (B) Day 15 post-infection with hemorrhages within myomeres and hydrocephalus, vcG001 low dose (scale bar = 5 mm). (C) Day 8 post-infection with hemorrhages along lateral line and within myomeres, vcG001 high dose (scale bar = 2 mm). (D) Day 15 post-infection with hydrocephalus, vcG002 high dose (scale bar = 2 mm)

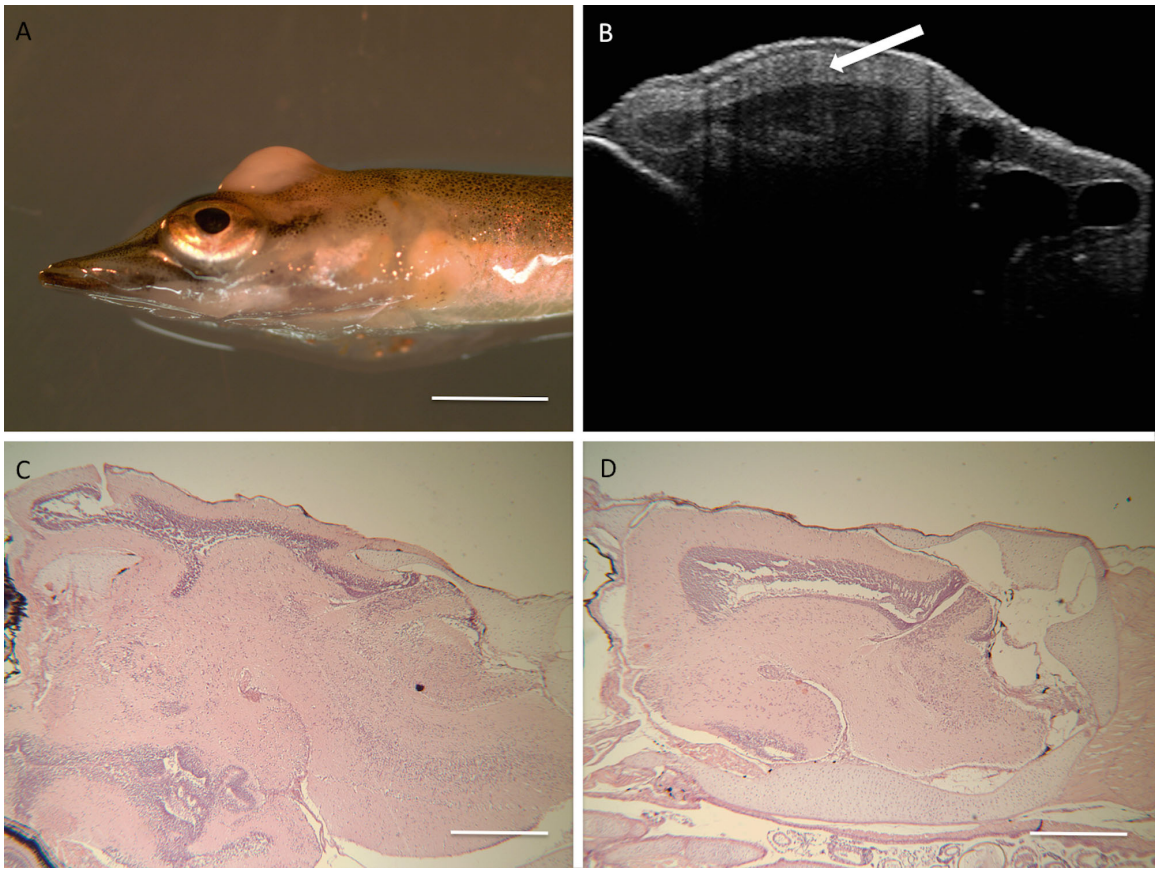

Fig. 3. Northern pike *Esox lucius* fry with hydrocephalus. (A) Day 15 post-infection with vcG002 high dose of viral hemorrhagic septicemia virus (scale bar = 5 mm). (B) Optical coherence tomography scan of a northern pike at day 21 post-infection with vcG060 low dose shows swelling of the optic tectum (arrow). (C) Histological section of the same specimen as (B) shows increased size of the brain compared with the age-matched control fish (scale bar = 500 µm). (D) Histological section of day 21 post immersion age-matched control fish (scale bar = 500 µm)

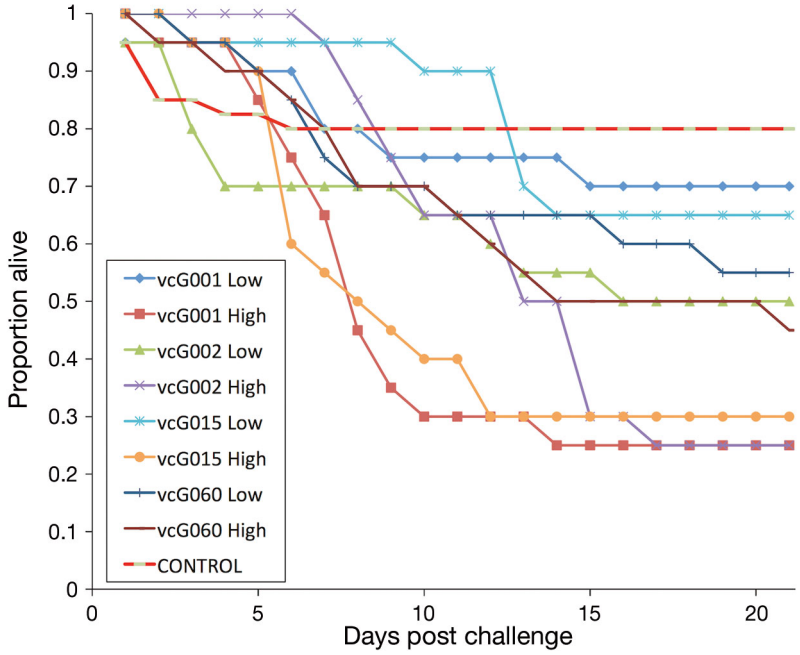

Fig. 4. Proportion of northern pike *Esox lucius* alive each day after immersion exposure to viral hemorrhagic septicemia virus. Groups include high- (5×10^5 PFU ml^{-1}) and low- (5×10^4 PFU ml^{-1}) dose for genotype IVb isolates vcG001, vcG002, vcG015, and vcG060. Variance among replicate tanks for each treatment was not significant in any case (data not shown), thus the mean cumulative mortalities for the replicates were used for this plot

distributed about zero and indicated no influential outliers (data not shown). Overall, there was a significant effect of dose on mortality that was consistent across the vcG types tested.

Comparison of viral load

The 2-way ANOVA was run with a main effect of strain (vcG), a main effect of dose, and the strain–dose relationship for the combined trunk and visceral tissue data for all dead and surviving fish. On analysis, the strain–dose relationship was not significant ($F = 0.925$, df = 1, $p = 0.337$), there was a difference in the viral load between the 2 doses ($F = 30.036$, df = 1, $p < 0.001$) and a difference in viral load between the strains ($F = 7.844$, df = 1, $p = 0.0057$). The 2-way ANOVA also was run with a main effect of strain, a main effect of dose, and the strain–dose relationship for the head and brain tissue data. The strain–dose relationship was again not significant ($F = 0.6400$, df = 1, $p = 0.425$), and there was no difference in the viral load between the strains ($F = 0.0097$, df = 1, $p = 0.921$). However, there was a difference in viral load between the 2 doses ($F = 31.889$, df = 1, $p < 0.001$). Next, a pairwise comparison between strains was performed with the Tukey multiple comparisons of means. The average viral load of the type strain vcG001 enumerated from the trunk and visceral tissues was significantly higher than the loads caused by vcG015 and vcG060 (ANOVA, Tukey's test; $p < 0.019$ and $p < 0.037$, respectively), not significantly different from vcG002 (ANOVA, Tukey's test; $p < 0.13$). No significant differences were noted in the viral load between the strains when the Tukey's test was conducted on the head and brain tissue mean values. Finally, in both the trunk and visceral tissue and head and brain tissue, there was a significant difference in the viral load of the dead ($F = 6.78$, df = 1, $p < 0.001$) versus the surviving ($F = 5.77$, df = 1, $p < 0.001$) northern pike.

Viral sequencing and genome analyses

Amplicons of expected size were synthesized by PCR with the 4 primer sets detailed in Schonherz et al. (2016), with the exception that fragment 1 appeared as a doublet with a couple of smaller bands as well, while fragments 2–4 were single bands (data not shown). Illumina sequencing was successful, generating complete genome sequences of 11 183 nt for each of the 4 Great Lakes VHSV isolates (deposited in GenBank as KY359354-57). An alignment of the 4 genome sequences showed no insertions or deletions. Each genome contained 6 major ORFs representing the known genes of VHSV in the expected order 3′-N-P-M-G-NV-L-5′ (Schütze et al. 1999). The genomes had identical non-translated 55 nt leader and 116 nt trailer sequences at the 3′ and 5′ termini, respectively, with the exception of a single polymorphism at nt 41 in the vcG014 leader. Eleven of the first 14 nt at the 3′ and 5′ genomic termini had reverse complimentarity as expected for all rhabdovirus genomes. Gene junction sequences were also conserved as previously reported, with single nt C or T intergenic regions, and consensus transcription end signals 5′-AGA TAG $(A)_7$, and transcription start signals 5′-GGC ACN NNT RT-3′ (Schütze et al. 1999). Among the 4 new genome sequences, the genome-wide genetic diversity was low, ranging from 0.19% (21/11 183 nt) for vcG001 and vcG002 to 0.38% (43/11 183 nt) for vcG015 and vcG060).

SNP analyses

Illumina sequencing identified 87 SNPs given a Phred quality call of 60 and a minimum coverage depth of 1000 in 67 of 87 contigs. These 87 SNPs varied among the 4 new VHSV IVb genome sequences and the reference MI03 sequence (GenBank GQ385941.1) and were spread throughout the genomes (Table 3), with a total of 7 in the N, 3 in the P, 5 in the N, 21 in the G, 1 in NV, and 36 in the L coding regions. There were also 13 SNPs in non-coding regions of the genomes, including the leader

Table 3. Single nucleotide polymorphisms (SNPs) detected in the coding (N = nucleoprotein, P = phosphoprotein, M = matrix protein, G = glycoprotein, NV = non-virion protein, and L = RNA polymerase) and non-coding (NC) regions across the 4 viral hemorrhagic septicemia virus IVb isolates relative to the reference VHSV IVb MI03 genome (GenBank: GQ385941.1). Twenty-six SNPs were non-synonymous (in parentheses)

Gene	vcG001	vcG002	vcG015	vcG060
N	0 (0)	1 (0)	2 (0)	4 (0)
P	0 (0)	0 (0)	1 (0)	2 (0)
M	0 (0)	2 (2)	1 (1)	2 (1)
G	0 (0)	6 (3)	7 (3)	8 (3)
NV	0 (0)	1 (1)	0 (0)	0 (0)
L	1 (1)	11 (3)	15 (6)	9 (2)
NC	2 (0)	2 (0)	5 (0)	5 (0)
Total	3 (1)	23 (9)	31 (10)	30 (6)

(1 SNP) and untranslated regions at gene junctions (12 SNPs). Our new sequence of the MI03 type strain differed from the previously reported MI03 reference genome sequence by 3 single nt substitutions; 2 in non-coding regions at nt 32 (leader) and nt 144 (N gene 5′ untranslated region), and the third at nt 660 in the L coding region. The finding of these SNPs in the vcG001 isolate may indicate mutations that occurred during the 2 cell culture passages prior to sequencing, though 2 of these non-coding changes were found in all 4 newly sequenced isolates. Silent (synonymous) substitutions outnumbered coding (non-synonymous) amino acid changes in all genes, with the exception of the M gene for vcG002 and vcG015. Transitional substitutions outnumbered transversions 71 to 16, with similar ratios within the different gene regions. Transitions are interchanges of 2-ring purines (A to G) or of 1-ring pyrimidines (C to T).

Non-synonymous changes were found in 26 VHSV IVb SNPs at 18 different positions within 4 of the 6 genes, including the M, G, NV, and L genes (Table 4). The amino acid changes in the 4 isolates sequenced were categorized according to a simple property-based scheme (Kelley et al. 2015). Eleven of the 18 changes stayed in the same group, while 7/18 changed properties (Table 4). Results from a series of dN/dS selection analyses on the 6 VHSV genes (Table 5) implied that diversifying selection potentially acts on only a small number of sites (4 total identified in all coding regions).

Table 4. Non-synonymous amino acid changes in 26 viral hemorrhagic septicemia virus single nucleotide polymorphisms at 18 different positions identified by gene and nucleotide numbers. The amino acid changes are colored according to a simple property-based scheme: (A, S, T, G, P: small/polar) are black, (M, I, L, V: hydrophobic) are green, (K, R, E, N, D, H, Q: charged) are red and (W, Y, F, C: aromatic + cysteine) are blue

Position	vcG001	vcG002	vcG015	vcG060
M 2313		T—I		T—I
M 2799			K—R	
M 2819		V—I		
G 3290		K—R	K—R	K—R
G 3398		G—D		G—D
G 3850			I—V	
G 4274		P—L	P—L	P—L
NV 4832		I—M		
L 5646			I—M	
L 5854		T—A		
L 6601	A—T			
L 7616		T—I	T—I	T—I
L 9220			L—F	
L 9276				M—I
L 9491			S—N	
L 9703			T—A	
L 9780		M—I		
L 10481			V—A	

The percentage of the called (non-reference) base in all the reads considered at that SNP position averaged 94.8, 97.8, and 98.0% for isolates vcG002, vcG015, and vcG060, respectively. Both sequencing errors (Illumina has an error rate of approximately 0.5%) and imperfect index sorting may have contributed to the less than 100% base calls for homogenous sites. As observed in previous VHSV sequence reports (Einer-Jensen et al. 2004, Thompson et al. 2011), some sites were heterogeneous, with more than one nt at the same position. Here heterogeneity was observed with one additional nt at positions 144 (vcG001), 2799 (vcG015), and 6601 (vcG001).

Phylogenetic relationship with global VHSV genome sequences

A phylogenetic tree estimating the relationships of the 4 new VHSV genome sequences with a set of with 24 full-length VHSV genomes available in GenBank is shown in Fig. 5. The GenBank genomes included VHSV isolates from genotypes I, II, III, and IVa, and the single available IVb reference genome previously reported for type strain MI03 (Ammayappan & Vakharia 2009; Table 6). The tree topology showed clear resolution of the 4 major VHSV genotypes with 100% bootstrap support in each case. The 4 new VHSV sequences all fell within genotype IV, in a strongly supported subclade with the previously reported MI03 IVb sequence, distinct from the next most closely related subgroup IVa. Thus the 4 complete genome sequences were clearly placed within the IVb subgroup, consistent with their geographic origin in the Great Lakes region.

Glycoprotein structure analysis

The 4 positions with amino acid changes (Table 4) did not appear to alter the predicted structure that modeled with 100.0% confidence to a template derived from an isolate of vesicular stomatitis virus (VSV, TaxId:11277). The Phyre2-based model of VHSV IVb glycoprotein used 385 residues (76% of

Table 5. Non-synonymous (dN)/synonymous (dS) analysis of viral hemorrhagic septicemia virus single nucleotide polymorphisms to identify codons potentially under positive (diversifying) or negative (purifying) selection (Murrell et al. 2012, 2013). Numbers represent the specific codons (counted from the starting Met of each gene) under either diversifying or purifying selection. In **bold** are sites (i.e. codons) under positive selection in both FUBAR (fast, unconstrained Bayesian approximation) and MEME (mixed effects model of evolution) analyses. The GARD tree topology was used in the analysis of all genes, and showed no recombination for any of them

Gene	FUBAR diversifying (pp > 0.9)	FUBAR purifying (pp > 0.9)	MEME diversifying (p < 0.1)
L	**1154**	243 codons	593, 847, 1092 **1154**, 1314, 1485
G	No sites	45, 216, 246, 471, 479	No sites
N	40,43	103, 105, 167, 191, 254, 297, 350	76
NV	No sites	No sites	No sites
M	186	12, 102	No sites
P	No sites	143	No sites

the sequence) to create a structure containing 3 rigid, mostly-beta domains and an alpha-helical region, acting as a conformational switch or 'fusion stalk' (data not shown).

DISCUSSION

Northern pike *Esox lucius* fry had moderate susceptibility to VHSV genotype IVb across all 4 isolates tested. The mortality of infected northern pike fry was lower when compared with juvenile muskellunge *Esox masquinongy* infected in the trials conducted the previous year with the same isolates, immersion doses, and conditions and where 100% muskellunge mortality was observed (Lewis et al. 2017). External lesions were seen in many of the northern pike mortalities, but no difference was observed in their prevalence or variety between the 4 sequence types. Imanse et al. (2014) also reported no difference in lesion prevalence with round goby *Neogobius melanostomus* infected with vcG001 and vcG002 isolates. The observation of hydrocephalus in

Fig. 5. Maximum likelihood phylogenetic tree showing bootstrap support. Roman numerals correspond to viral hemorrhagic septicemia virus (VHSV) genotype groups. Bootstrap values are 100% at each node. The origin of the representative VHSV isolates used for phylogenetic analysis is shown in Table 6

Table 6. Representative viral hemorrhagic septicemia virus (VHSV) isolates from each genotype (I, II, III, IVa, IVb) used for phylogenetic analysis with isolate details retrieved from GenBank

Isolate code	Genotype	Year of isolation	Origin	Host species	Accession number
SE-SVA143D	I	1998	Sweden	Rainbow trout	AB839745
DK_4p37	I	1997	North Sea	Blue whiting	AY546580
SE-SVA1033	I	2000	Kattergat	Rainbow trout	AY546623
KRRV9601	I	1996	Japan	Japanese flounder	AB672614
96_43	I	1996	English Channel	Herring	AF143862
Cod Ulcus	I	1979	United Kingdom	Cod	Z93414
Y_Fil3	I	1998	France	Rainbow trout	Y18263
DK_3592B	I	1986	Denmark	Rainbow trout	AY356632
French_07-71	I	1971	France	Rainbow trout	AJ233396
14_58	I	1999	France	Rainbow trout	AF14386
23_75	I	1975	France	Brown trout	FN665788
Hededam	I	1972	Denmark	Rainbow trout	Z93412
DK1p49	II	2014	Denmark	Herring	KM244767
FA281107	III	2007	Norway	Rainbow trout	EU481506
4p168	III	1997	Skagerrak	Herring	AY546582
GH40	III	2014	Spain	Greenland halibut	KM244768
FP_VHS2010	IVa	2010	South Korea	Olive flounder	KP334106
JF_09	IVa	2009	South Korea	Olive flounder	KM926343
KJ2008	IVa	2008	South Korea	Olive flounder	JF792424
FYeosu05	IVa	2005	South Korea	Olive flounder	KF477302
PORV	IVa	2005	China	Japanese flounder	KC685626
JF00ehi1	IVa	2000	Japan	Japanese flounder	AB490792
KRRV9822	IVa	2000	Japan	Japanese flounder	AB179621
GQ385941.1	IVb	2003	USA	Muskellunge	GQ385941
MI03 (VHSV_01)	IVb	2003	USA	Muskellunge	KY359354 FPL2006-005
(VHSV_02)	IVb	2006	USA	Round goby	KY359355 FPL2013-002
(VHSV_13)	IVb	2013	USA	Gizzard shad	KY359357 FPL2014-001
(VHSV_14)	IVb	2014	USA	Gizzard shad	KY359356

16/84 (19%) of the mortalities and 1 of the day-21 survivors was unexpected, given that this external sign was not seen in the previous muskellunge trial (Lewis et al. 2017). However, Bootsma (1971) did observe similar severe swelling with northern pike fry, shown later to be caused by pike fry rhabdovirus (de Kinkelin et al. 1973). Intracranial hemorrhages have frequently been observed in round goby infected by immersion and intraperitoneal injection (Cornwell et al. 2014, Imanse et al. 2014). The faster time to death with juvenile muskellunge combined with a thicker cranium at that life stage could explain the different presentation seen in northern pike.

The viral loads of the 4 IVb isolates from the trunk and visceral tissues in this trial with northern pike differed from a previous trial with muskellunge (Lewis et al. 2017), with the type strain MI03 (vcG001) having a higher viral load than the vcG015 and vcG060 strains, though the 2006 isolate (vcG002) produced a viral load in northern pike that was not significantly different from the 2003 MI03 type strain. Imanse et al. (2014) did find a difference in viral load when round goby were exposed by immersion to low doses of the type strain (vcG001) and a 2010 (vcG002) VHSV IVb isolate. The time to death in this northern pike trial was not significantly different among the 4 strains tested. A significant number of northern pike survivors (41/72) remained infected in both the head and brain and trunk and visceral organs at the end of the 21-d trial when measured by RT-qPCR. This is similar to what was observed in goldfish *Carassius auratus* in a previous challenge study with the MI03 type strain (Getchell et al. 2015). Brudeseth et al. (2002) found VHSV in rainbow trout *Oncorhyncus mykiss* brains as early as day 5 post-infection, and virus present in the neurons and neuroglia cells. Whether the viral levels in northern pike would persist long term in tissues such as brain, as others have

suggested, would require much longer holding times for survivors (Neukirch & Glass 1984, Neukirch 1986, Hershberger et al. 2010, Lovy et al. 2012, Ito et al. 2016). Olson (2013) observed a significant VHSV IVb load in the brain of yellow perch *Perca flavescens*, particularly in the later stages of infection. Snow et al. (2000) suggested that the brain was a potential site for virus to persist and escape immunodetection, which would have significant implications in the maintenance of viral reservoirs in wild fish. The 43% (31/72) of the survivors that yielded negative RT-qPCR results may have been infected, but below the limit of detection (~100 copies; Hope et al. 2010), uninfected, or fish that cleared the virus.

Observation of morbidity and mortality in VHSV immersion trials has clearly varied in other susceptibility trials with VHSV IVb. Grice et al. (2015) suggested that this variability could be due to strain differences in the host, while others point to genotype and experimental protocol differences. Genotypic variants may allow VHSV to adapt to a variety of new environments and fish species (Pierce & Stepien 2012). Differences in virulence of virus genotype within species have been reported for yellow perch, Pacific herring *Clupea pallasii*, koi *Cyprinus carpio koi*, and salmonids, in which genotype Ia is highly virulent compared with genotypes IVa, IVb, and IVc (Emmenegger et al. 2013). Cornwell et al. (2014) found there was no difference in the observed diversity of sequence types of virus from round goby that tested positive during times of low or high prevalence, or during a confinement-induced laboratory epidemic. Others have reported sequence diversity within a single host (Einer-Jensen et al. 2004, Thompson et al. 2011). In 2007, extensive gizzard shad *Dorosoma cepedianum* mortalities in Dunkirk Harbor, NY occurred throughout March. Both vcG001 and vcG002 were typed from these specimens (Thompson et al. 2011). The 2014 Dunkirk gizzard shad isolate tested and sequenced in this study was typed as vcG015 and had 10 amino acid changes (31 nt changes) from the vcG001 type strain across the whole genome. The vcG015 sequence type had also been detected from round goby at Cape Vincent, NY and Selkirk, NY in 2009 (Cornwell et al. 2012).

This study is unique in 2 ways; simultaneously testing virulence of 4 VHSV IVb isolates using a northern pike model and deep sequencing these isolates on an Illumina MiSeq platform. While there were few discernible differences in virulence between isolates vcG001, vcG002, vcG015, and vcG060, other than a higher viral load for the type strain, every bit of evidence of phenotypic differences between sequence types of VHSV genotype IVb in the Great Lakes helps those studying the evolution of these new pathogens. There were 26 non-synonymous changes located at 18 different sites in the genomes when the SNP data from all the isolates were combined. Only 1 of these amino acid changes was found in the central portion of the G gene, a location used previously to characterize VHSV genetic diversity (Thompson et al. 2011, Stepien et al. 2015). Relative to the MI03 type strain isolated in 2003, the 2 most recent isolates that caused gizzard shad mortalities in 2013 and 2014 had slightly higher numbers of SNPs (30 and 31 SNPs) than the 2006 vcG002 isolate (23 SNPs), but the numbers of non-synonymous changes were similar (Table 3). When Thompson et al. (2011) analyzed 108 early isolates, 98% of them differed from each other by no more than a single nucleotide in the central region of the G gene. This was largely due to a high prevalence of vcG001 and vcG002 type isolates, which differed by only 1 nucleotide in the vcG region (0.15% nt divergence). The full-length genome sequences of the vcG001 and vcG002 isolates here revealed a slightly higher genome-wide nt divergence of 0.19%, suggesting that regions outside the central G region must have higher average divergence. When the most recent 2014 isolate (vcG015) was sequenced, 31 SNPs were identified in the complete genome, a 0.28% pairwise divergence from the MI03 type strain, and the most divergent sequences in this study were the 2 recent isolates vcG015 and vcG060 (0.38%). This provides evidence for increasing genetic diversification within sub-genotype IVb over time, although the overall level of divergence is still very low compared with the known global VHSV nt divergence of nearly 20% between viruses in genotypes I and IV. Most VHSV IVb genotypes have only been distinguished from the type strain by nucleotide sequence differences occurring in the genes coding for the viral nucleocapsid and glycoproteins, though Stepien et al. (2015) did sequence fragments of the NV, M, and P genes in 12 isolates from the Great Lakes area. We examined predicted fusion protein models using the 4 VHSV IVb glycoprotein genes sequenced in this study, finding a similarity of structure to VSV that others had previously reported (Einer-Jensen et al. 1998, Walker & Kongsuwan 1999). Researchers have attempted to identify variations in regions of the VHSV genome that are associated with phenotypic changes such as virulence increase *in vivo* (Béarzotti et al. 1995, Betts & Stone 2000, Snow & Cunningham 2000, Imanse et al. 2014). Most recently, Ito et al. (2016) suggested that substitutions of amino acids in positions 118–123 of the

nucleoprotein are candidates for being related to virulence of VHSV genotype III in rainbow trout. A single amino acid mutation (I1012F) of the L gene can change the virulence to rainbow trout gill epithelial cells (Kim et al. 2014). Though the G and NV genes also have been the focus of these research efforts, Einer-Jensen et al. (2014) did not detect virulence differences using reverse genetics to create chimeric viruses and manipulate these 2 genes from high- and low-virulence isolates. With the exception of vcG015 sharing the V1802A substitution in the L gene of VHSV genotype III NO-2007-50-385, to our knowledge, none of the published VHSV gene substitutions were found in the genotype IVb isolates sequenced here.

Our analyses suggest that positive (i.e. diversifying) selection does not seem to be a main evolutionary force behind the diversification of genotype IVb. Instead, most codons are evolving under either a neutral or a purifying selection model. As probably expected, the L coding gene is the one gene that shows the highest excess of synonymous substitutions. Interestingly, no positively selected codons were identified in the glycoprotein gene, which seems to have diversified under a (nearly) neutral model.

Which amino acid substitutions play the key roles for VHSV virulence remains elusive (Ito et al. 2016). Further genomic studies should continue to examine complete sequences of more VHSV IVb isolates to identify the key sequence variations driving virulence changes. More work will be needed to identify whether the mutations discovered in these gizzard shad isolates caused the reduction in viral load seen in this study. Finding the specific amino acid and protein changes influencing variant pathogenesis will help drive vaccine development and future control measures.

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