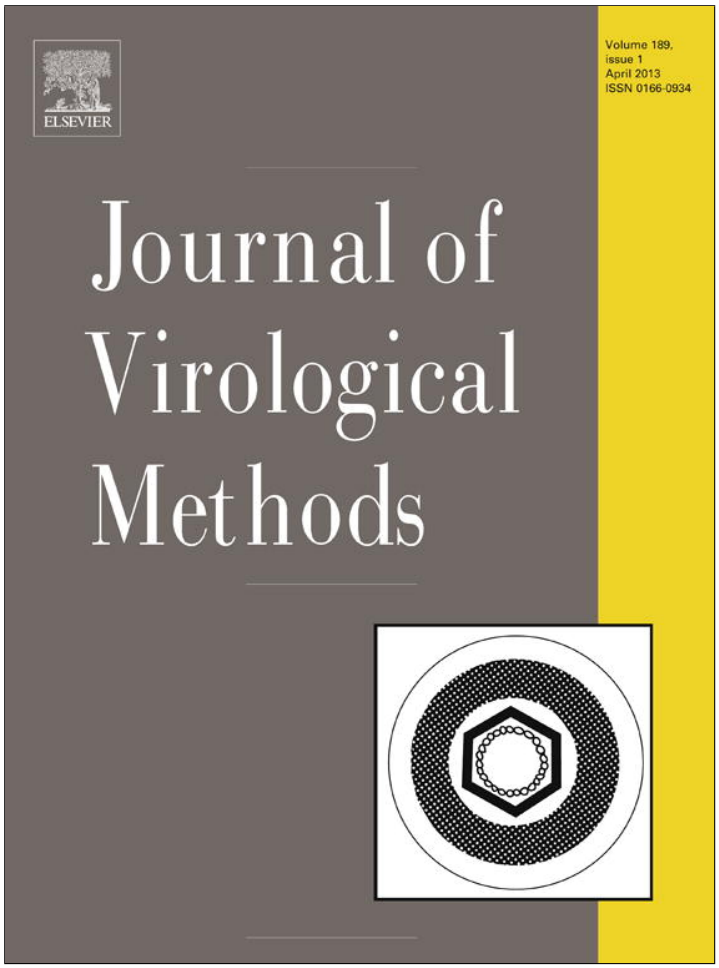

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A new StaRT-PCR approach to detect and quantify fish Viral Hemorrhagic Septicemia virus (VHSv): Enhanced quality control with internal standards

Lindsey R. Pierce[a], James C. Willey[b,1], Erin L. Crawford[b], Vrushalee V. Palsule[a], Douglas W. Leaman[c], Mohamed Faisal[d], Robert K. Kim[d], Brian S. Shepherd[e], Lauren M. Stanoszek[b], Carol A. Stepien[a,*]

[a] Great Lakes Genetics/Genomics Laboratory, Lake Erie Center and Department of Environmental Sciences, The University of Toledo, 6200 Bayshore Road, Toledo, OH 43616, United States
[b] Department of Medicine, The University of Toledo, 3100 Arlington Avenue, Toledo, OH 43614, United States
[c] Department of Biology, The University of Toledo, 2801 West Bancroft Street, Toledo, OH 43606, United States
[d] Aquatic Animal Health Laboratory, Michigan State University, 174 Food Safety and Toxicology Building, East Lansing, MI 48824, United States
[e] University of Wisconsin at Milwaukee, School of Freshwater Sciences/ARS/USDA, 600 East Greenfield Avenue, Milwaukee, WI 53204, United States

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A B S T R A C T

Viral Hemorrhagic Septicemia virus (VHSv) causes one of the world's most important finfish diseases, killing >80 species across Eurasia and North America. A new and especially virulent strain (IVb) emerged in the North American Great Lakes in 2003, threatening fisheries, baitfish, and aquaculture industries. Weeks-long and costly cell culture is the OIE and USDA-APHIS approved diagnostic. A new Standardized Reverse Transcriptase Polymerase Chain Reaction (StaRT-PCR) assay that uniquely incorporates internal standards to improve accuracy and prevent false negatives was developed and evaluated for its ability to detect and quantify VHSv. Results from StaRT-PCR, SYBR® green real time qRT-PCR, and cell culture were compared, as well as the effects of potential PCR inhibitors (EDTA and high RNA). Findings show that StaRT-PCR is sensitive, detecting a single molecule, with 100% accuracy at six molecules, and had no false negatives. In comparison, false negatives ranged from 14 to 47% in SYBR® green real time qRT-PCR tests, and 47–70% with cell culture. StaRT-PCR uniquely controlled for EDTA and RNA interference. Range of VHSv quantitation by StaRT-PCR was 1.0×10^0–1.2×10^5 VHSv/10^6 *actb1* molecules in wild caught fishes and 1.0×10^0–8.4×10^5 molecules in laboratory challenged specimens. In the latter experiments, muskellunge with skin lesions had significantly more viral molecules (mean = 1.9×10^4) than those without (1.1×10^3) ($p < 0.04$). VHSv infection was detected earlier in injection than in immersion challenged yellow perch (two versus three days), with molecule numbers in both being comparable and relatively consistent over the remaining course of the experiment. Our results show that the StaRT-PCR test accurately and reliably detects and quantifies VHSv.

1. Introduction

1.1. VHSv characteristics and spread

Viral Hemorrhagic Septicemia virus (VHSv) is one of the world's most serious fish pathogens, killing over 80 marine and freshwater species (including trout, salmon, and perch) across the Northern Hemisphere (Faisal et al., 2012), yet has lacked a rapid and accurate diagnostic test. The virus is a negative-sense, single stranded RNA *Novirhabdovirus* of ~12,000 nucleotides, with six open reading frames of 3′N-P-M-G-Nv-L′5 (Ammayappan and Vakharia, 2009). A new and especially virulent VHSv substrain (IVb) emerged in the Laurentian Great Lakes of North America in 2003, causing massive fish kills (Elsayed et al., 2006; Faisal et al., 2012) that have threatened the fisheries, aquaculture, baitfish, and tourism industries (Leighton, 2011).

Transmission of VHSv occurs via fish waste, reproductive fluids, and skin secretions. Its viral particles can live up to 13 days in the water (Hawley and Garver, 2008) and are transported via boating, ballast water, fishing tackle, and animals – e.g., amphipod crustaceans, leeches, turtles, and birds (Faisal and Schulz, 2009; Bain et al., 2010; Faisal and Winters, 2011; Goodwin and Merry, 2011). Clinical signs of infection vary, ranging from erratic swimming, exophthalmia (bulging eyes), distended abdomens, to extensive external/internal bleeding (Winton and Einer-Jensen, 2002). Since November 2006, the eight U.S. states (Illinois, Indiana, Ohio, Pennsylvania, Michigan, Minnesota, New York, Wisconsin) and two Canadian provinces (Ontario and Quebec) that surround the Great

* Corresponding author. Tel.: +1 419 530 8362; fax: +1 419 530 8399.
E-mail address: carol.stepien@utoledo.edu (C.A. Stepien).
[1] Technical questions regarding the StaRT-PCR method should be requested to James Willey (james.willey2@utoledo.edu).

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Lakes have required that fish are certified as VHSv-free prior to interstate transport (Aquatic Invasive Species Action Plan, 2011), for which the Office International des Epizooties (OIE) recommends a month long cell culture process (OIE, 2009).

VHSv (originally called "*Niwensckwellzing*") first was described from European salmonid aquaculture (Shäperclaus, 1938), and was isolated in 1962 from infected rainbow trout (*Oncorhynchus mykiss*) (Einer-Jensen et al., 2004). Four genetically distinct strains (I–IV) and various substrains have been recognized (Snow et al., 1999; Einer-Jensen et al., 2004), whose phylogenetic and biogeographic relationships recently were analyzed by Pierce and Stepien (2012). Strains I–III are found in Europe, where strain I infects >13 freshwater species including rainbow and brown trout (*Salmo trutta*). Strain II comprises a tight genetic cluster (Pierce and Stepien, 2012), which primarily infects Pacific herring (*Clupea pallasii*) in the Baltic Sea and Finland Archipelago (Gadd et al., 2011). Strain III is distributed peripherally to strain I, infecting Atlantic cod (*Gadus morhua*), European eel (*Anguilla anguilla*), haddock (*Melanogrammus aeglefinus*), Norway pout (*Trisopterus esmarki*), Pacific herring, rainbow and brown trout, turbot (*Scophthalmus maximus*), whiting (*Merlangius merlangus*), and others. Strain IV was described in 1988 from the North American Pacific Northwest (substrain a), where it is found in a wide variety of marine fish species (including chinook salmon (*Oncorhynchus tshawytsha*), coho salmon (*Oncorhynchus kisutch*), Pacific cod (*Gadus macrocephalus*), Pacific sardine (*Sardinops sagax*), and smelt (*Thaleichthys pacificus*)), with a few occurrences in Japan and Korea in black seabream (*Spondyliosoma cantharus*) and olive flounder (*Paralichthys olivaceus*) (Kim and Faisal, 2011). Substrain IVb was identified in the Great Lakes (Lake St. Clair) basin from an adult muskellunge (*Esox masquinongy*) collected in 2003 (MI03GL; Elsayed et al., 2006). IVb has spread to infect 31 freshwater fish species across all five of the Great Lakes (Thompson et al., 2011), with some isolates found in invertebrates, indicating that they might serve as transmission vectors (Faisal and Schulz, 2009; Faisal and Winters, 2011).

1.2. Need for a new VHSv diagnostic test

Screening methods for VHSv that are approved by the World Organization for Animal Health (OIE, 2009), and the Fish Health Section of the U.S. Fish and Wildlife Service and the American Fisheries Society (2010) are based on identification via cell culture, followed by confirmation either with reverse transcriptase PCR or serologically via an indirect fluorescent antibody test or an enzyme-linked immunosorbent assay. Although those methods readily detect high concentrations of the virus, they may fail to detect low levels of virus in carrier fish. Notably, fish have been shown to shed virus for up to 15 weeks post infection (Kim and Faisal, 2012).

Cell culture identification and subsequent confirmation is labor intensive, time consuming (up to four weeks), and less sensitive than direct PCR-based detection, with false negative rates reported up to 95% (López-Vázquez et al., 2006; Miller et al., 1998; Winton and Einer-Jensen, 2002). For fish farms, hatcheries, and baitfish operators, a month-long holding period for viral detection leads to economic loss and possible viral spread. Additionally, cell culture facilities typically "pool" samples (i.e., tissues from several fish samples are homogenized together), which dilutes the number of viral particles and circumvents pinpointing exactly which samples are VHS positive. Further, cell culture identification methods, such as plaque assay, may not precisely quantify the number of viral particles or allow determination of the amount leading to infection.

Non-lethal techniques to detect neutralizing glycoprotein (G) gene antibodies in fish blood have been developed, with experiments showing that VHSv remained detectable for ≥90 days post exposure (Millard and Faisal, 2012). However, that antibody approach relied on confluent cultured cells followed by a 6–7 day incubation and an optimum virus concentration for stimulating antibody production (which remains to be characterized). Thus, that weeks-long test may be insufficient for detecting low amounts of VHSv in carrier fish. An assay using monoclonal antibodies by Ito et al. (2012) was developed to identify VHSv strains and substrains. However, that method also used cultured cells and relied on generation of specific antibodies, which are lengthy and costly procedures.

Quantitative real-time PCR (qRT-PCR) approaches have greater sensitivity and reduced detection time in comparison to cell culture and plaque assays (Bruchhof et al., 1995; Miller et al., 1998; Gillou et al., 1999). Several qRT-PCR assays for VHSv have been developed, including: Chico et al. (2006), López-Vázquez et al. (2006), Liu et al. (2008), Matejusova et al. (2008), Cutrín et al. (2009), Hope et al. (2010), Garver et al. (2011), Jonstrup et al. (2013), and Phelps et al. (2012); however, all of these have accuracy limitations. Just three of those tests quantified VHSv levels: Liu et al. (2008), Hope et al. (2010), and Garver et al. (2011). For example, Liu et al. (2008) distinguished to 140 viral copies of a single VHSv type (Ia; isolate Fil3); however, it remains unknown whether their test method, which was based on the G-gene, would work on other VHSv variants. Other assays used the nucleoprotein (N) gene (Chico et al., 2006; Cutrín et al., 2009; Garver et al., 2011; Jonstrup et al., 2013; López-Vázquez et al., 2006; Matejusova et al., 2008; OIE, 2009), including a test by Hope et al. (2010) that reliably distinguished to 100 viral copies. However, higher amounts of RNA (1 μg) led to a 20-fold reduction in their PCR amplification signal, with further signal decline at RNA inputs of 4–8 μg (Hope et al., 2010). That decline likely was due to interference and/or reagent carry over and would be prone to generate false negative results. The assay by Garver et al. (2011) detected to 100 viral copies, based on results from several laboratories in blind experiments. Jonstrup et al. (2013) reported increased sensitivity compared to other assays because they observed amplification at a lower cycle threshold (C_t; the number of cycles at which the fluorescence exceeded the threshold). Their method was evaluated for all four VHSv strains and 79 isolates, yet did not discern significantly more VHS positives than cell culture ($\chi^2 = 0.10$, $df = 1$, NS; statistical analyses performed in the present study using their results). Their findings suggested a large proportion of false negatives (Jonstrup et al., 2013).

1.3. The StaRT-PCR method and study objectives

To date, qRT-PCR and plaque/immunological assays for VHSv have lacked Internal Standards (IS) to control for interfering substances and false negative results, which may lead to misdiagnosis and potential viral spread. The present study thus developed a new PCR-based test for VHSv, which incorporates a Standardized Mixture of Internal Standards (SMIS) in a Standardized Reverse Transcriptase Polymerase Chain Reaction (StaRT-PCR); this method follows the StaRT-PCR approach outlined by Crawford et al. (2002), Willey et al. (2004), and Canales et al. (2006).

StaRT-PCR is a form of competitive PCR (Gilliland et al., 1990; Celi et al., 1993) that measures expression of each gene relative to a known number of copies of a synthetic competitive template IS within a SMIS (Willey, 2004). An IS is constructed for each target gene (i.e., VHSv N-gene) and for one or more reference genes (i.e., genes that are expressed at a relatively consistent level across many tissues and conditions), which are combined into a SMIS. The SMIS is loaded into each reaction (rxn). This approach also controls for interfering substances, such as PCR inhibitors, and prevents false negative results. For example, if the IS PCR product is not observed, it will be interpreted as a failed test and not as valid assessment for the absence of VHSv. StaRT-PCR is designed to yield rapid, reproducible, standardized, and quantified measures from several genes simultaneously.

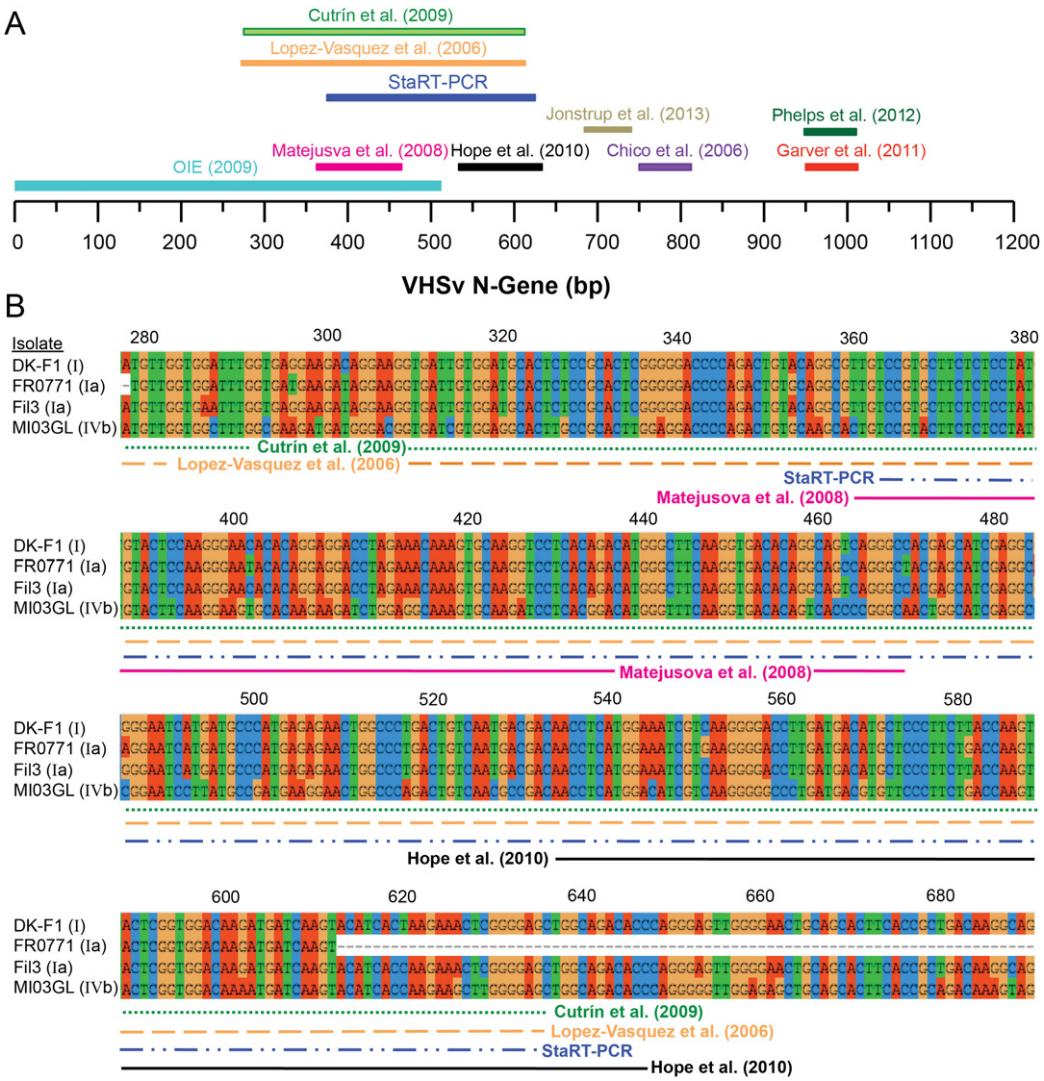

Fig. 1. Nucleotide map of the VHSv N-gene, showing locations of our StaRT-PCR primers in relation to those from other studies (Chico et al., 2006; Cutrín et al., 2009; Garver et al., 2011; Hope et al., 2010; Jonstrup et al., 2013; López-Vázquez et al., 2006; Matejusova et al., 2008; OIE, 2009; Phelps et al., 2012).

The research aim of the present study was to evaluate the performance and accuracy of the newly developed StaRT-PCR VHSv test that incorporates synthetic IS, in comparison to conventional qRT-PCR-based assays (e.g., SYBR® green qRT-PCR) that lack IS, and cell culture. Experiments were conducted to test the ability of StaRT-PCR to discern and quantify VHSv from (A) pellets of cells infected in vitro, (B) wild caught fishes, (C) fish (muskellunge and yellow perch (*Perca flavescens*)) that were experimentally challenged with VHSv, and (D) laboratory altered samples containing various PCR inhibitors. The latter included both exogenous inhibitors (e.g., ethylenediaminetetraacetic acid (EDTA)) and endogenous inhibitors from high levels of RNA in reverse transcription. The experiments were designed to test the hypotheses whether StaRT-PCR (1) is VHSv-specific, (2) accurately measures the amount of VHSv in infected fish cells, (3) controls for PCR inhibitors, (4) accurately and reliably diagnoses and quantifies VHSv in field and laboratory fish samples, and (5) has greater sensitivity and reliability than cell culture or other qRT-PCR methods. Laboratory challenge experiments also were conducted to determine whether the number of VHSv molecules significantly differs in challenged fish (6) with or without clinical signs of infection, (7) over the course of early infection (to day six), and/or (8) between immersion and injected challenged individuals. These evaluations provided examples of the potential applications of the StaRT-PCR VHSv test.

2. Methods

2.1. Design of the StaRT-PCR test

Primers and the IS designed for the StaRT-PCR VHSv test targeted the central portion of the VHSv N-gene (Fig. 1). The N-gene expresses RNA transcripts most abundantly in rhabdovirus infections (Chico et al., 2006) and is relatively conserved across species due to its RNA-binding function (Hope et al., 2010). Other studies showed that primers targeting the VHSv N-gene were more efficient and sensitive than those targeting the G-gene (Chico et al., 2006; Cutrín et al., 2009). In the present study, N-gene sequences were aligned from all VHSv variants, related *Novirhabdoviruses*, and other viruses from NIH GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using the BLAST procedure (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure that the designed primers did not recognize other viruses. VHSv-specific inter-variant homologous sequences then were targeted for primer design, and Oligo software (http://www.oligo.net/) was used to select primers based on the absence of stable duplex formation,

Table 1
Sequences and PCR parameters of primers and Internal Standards (IS) used for StaRT-PCR. F, forward; R, reverse; CT, competitive template.

Primer and IS	Nucleotide position	Sequence (5′-3′)	T_m (°C)	GC%	Product length (bp)
N-gene					
VHSv F5	362–383	GTC CGT GCT TCT CTC CTA TGT	66.9	52.4	273
VHSv R5	614–635	TCC CCG AGT TTC TTA GTG ATG	67.6	47.6	273
Reference gene primers					
actb1 F1	620–640	TTG GCT GGC CGT GAC CTC AC	77.6	65.0	190
actb1 R1	790–810	GCA GCT CGT AGC TCT TCT CC	67.9	60.0	190
ef1a F1	239–259	ACC ACC GGC CAT CTG ATC TA	71.2	55.0	220
ef1a R1	440–459	TGT GTC CAG GGG CAT CAA T	70.9	52.6	220
18srRNA F1	4513–4533	AGT ACA CAC GGC CGG TAC AG	69.5	60.0	269
18srRNA R1	4762–4786	GGG CAG ACA TTC GAA TGA GA	69.6	50.0	269
Reverse transcription efficiency primers					
ERCC 113 F	116–136	TTG GAT CAG TGG GAA GTG CT	69.1	50.0	130
ERCC 113 R	226–246	GGG GCT CGA AAG GTA CTA GG	68.5	60.0	130
ERCC 171 F	153–173	AAG CTG ACG GTG ACA AGG TT	68.2	50.0	118
ERCC 171 R	251–271	TCG CAG TTT TCC TCA AAT CC	68.6	45.0	118
Internal standards					
VHSv CT5	543–585	TCC CCG AGT TTC TTA GTG ATG CAA GGT CCC CTT GAC GAT TTC	89.9	50.0	223
actb1 CT1	747–787	GCA GCT CGT AGC TCT TCT CCG CCC ATC TCC TGC TCG AAG T	91.9	60.0	167
ef1a CT1	389–427	TGT GTC CAG GGG CAT CAA TTC CAG AGA GCG ATG TCG AT	91.5	52.6	188
18srRNA CT1	4671–4711	GGG CAG ACA TTC GAA TGA GAA TGA GAG AGG CAC CCC GCA TGG GTT T	94.3	57.5	198
ERCC 113 CT	116–218	TTG GAT CAG TGG GAA GTG CTC ACG CGC GGA GCC CAC TGG GCG AAC AGC AAC GTT ATA ACG GCC ACT CAG TGG TTC GTC ACG CCC TAG TAC CTT TCG AGC CCC	87.0	59.8	102
ERCC 171 CT	153–250	AAG CTG ACG GTG ACA AGG TTT CCC CCT AAT CGA GAC GCT GCA ATA ACA CAG GGG CAT ACA GTA ACC AGG CAA GAG TTG GAT TTG AGG AAA ACT GCG A	82.5	49.5	97

low likelihood of false priming sites, and an optimal annealing temperature of 58 °C. Primer sequences are given in Table 1, and their locations and relationships to those from other assays are shown in Fig. 1.

Primer sets and IS for three fish reference genes – β-actin (*actb1*), elongation factor 1 alpha subunit (*ef1a*), and 18S ribosomal RNA (*18srRNA*) – were developed to ensure accurate diagnosis and quantitation under a variety of PCR and sample conditions (Table 1). Reference gene sequences for primers and IS were selected from conserved regions across 10 VHSv-affected fish species (five sport fish species: yellow perch, smallmouth bass (*Micropterus dolomieu*), round goby (*Neogobius melanostomus*), freshwater drum (*Aplodinotus grunniens*), and walleye (*Sander vitreus*) and five baitfish species: emerald shiner (*Notropis atherinoides*), golden shiner (*Notemigonus crysoleucas*), fathead minnow (*Pimephales promelas*), spottail shiner (*Notropis hudsonius*), and alewife (*Alosa pseudoharengus*)). Each reference gene was measured to determine whether it was expressed at a consistent level in VHSv infected and non-infected fish, to validate its use as a control for variation in loading of sample into PCR. In these experiments, the reference genes were measured for five VHS positive and five negative control fish samples (muskellunge and yellow perch).

The IS for the VHSv N-gene and each of the three reference genes were prepared following the method of Celi et al. (1993) (see their Fig. 1A). First, primers were designed and synthesized for each of the four genes. Then, a modified reverse primer, called the IS primer, was constructed such that the 3′ end had sequence homology to a region between the forward and reverse primers and the 5′ end of the IS primer was identical to the reverse primer (Table 1). PCR extension only occurred from the sequence annealed at the 3′ end. This resulted in a PCR product for the IS that was shorter than the native target (NT) sequence. This difference in size enabled electrophoretic size separation and quantitation of IS and NT. The IS primer was used only to create the IS and was not used in the StaRT-PCR assays.

To develop the SMIS, each IS was generated by separate PCR amplification of fish cDNA using the forward primer (19–21 bp; Table 1) and the IS primer (38–42 bp; Table 1) in five 10 μl PCR replicates containing 1 μl (0.05 μg) of each primer, 0.5 U GoTAQ polymerase (Promega, Madison, WI), 1 μl 10× $MgCl_2$ PCR buffer, 1 μl 0.2 mM dNTPs, 5.5 μl RNase-free water, and 1 μl cDNA resulting from a 90 μl reverse transcription rxn containing 1 μg of VHSv positive fish RNA. Each of the five replicate samples then were transferred to LightCycler® capillaries (Roche, Indianapolis, IN) and cycled on a Rapid Cycler 2 (Idaho Technology, Inc., Salt Lake City, UT), with each cycle being 5 s at 94 °C, 10 s at 58 °C, and 15 s at 72 °C, and a slope of 9.9 for rapid temperature change between cycles (35 cycles total). PCR products from the five replicates then were combined, separated by electrophoresis on a 2% low melting agarose gel (Fisher Scientific, Fair Lawn, NJ) containing 5 μl ethidium bromide (10 mg/ml) per 100 ml 1X TRISAcetate EDTA buffer, and visualized on a UV transilluminator. The band corresponding to each IS was excised from the gel and purified using a Qiaquick Gel Extraction Qiagen Kit (Qiagen, Germantown, MD). Molarities were calculated by analysis of 1 μl of the purified products on a Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA) and concentrations were converted into molecules using the below formula (1):

$$\frac{[\text{Moles/Liter}]}{[1.0 \times 10^6\ \mu\text{l/L}]} \times [6.0 \times 10^{23}\ \text{molecules/Mole}] = \text{molecules}/\mu\text{l} \quad (1)$$

A Standardized Mixture of Internal Standards (SMIS) was created by mixing the individual gene IS together, as shown in Table 2, to enable measurement of expression across a range of possible numbers of target molecules. To obtain SMIS "A", a stock was created such that the IS for *18srRNA* was at 10^{-10} M, the IS for *actb1* was at 10^{-11} M, the IS for *ef1a* was at 10^{-10} M, and the IS for VHSv was at 10^{-10} M. A separate mixture of the three reference gene IS, which did not contain VHSv IS, was created with *18srRNA* at 10^{-10} M, *actb1* at 10^{-11} M, and *ef1a* at 10^{-10} M. This reference gene IS mixture was used in a 10-fold serial dilution of the SMIS "A" stock to create stocks for SMIS B-H (row 1 of Table 2). A solution of 0.1 ng/μl yeast tRNA carrier (Invitrogen, Carlsbad, CA) was used in all dilutions to prevent adherence of the negatively charged IS molecules to the plastic tube or pipette tip surfaces/diluents. Stock SMIS then was diluted down serially with carrier solution (rows 2–8 of Table 2) to generate a range of concentrations and enable measurement over several orders of magnitude.

Table 2
StaRT-PCR SMIS (Standardized Mixture of Internal Standards) dilution mixtures of the four IS (Internal Standards): *18srRNA/actb1/ef1a/VHSv*. Values are reported as 10^X M.

A	B	C	D	E	F	G	H
−10/−11/−10/−10	−10/−11/−10/−11	−10/−11/−10/−12	−10/−11/−10/−13	−10/−11/−10/−14	−10/−11/−10/−15	−10/−11/−10/−16	−10/−11/−10/−17
−11/−12/−11/−11	−11/−12/−11/−12	−11/−12/−11/−13	−11/−12/−11/−14	−11/−12/−11/−15	−11/−12/−11/−16	−11/−12/−11/−17	
−12/−13/−12/−12	−12/−13/−12/−13	−12/−13/−12/−14	−12/−13/−12/−15	−12/−13/−12/−16	−12/−13/−12/−17		
−13/−14/−13/−13	−13/−14/−13/−14	−13/−14/−13/−15	−13/−14/−13/−16	−13/−14/−13/−17			
−14/−15/−14/−14	−14/−15/−14/−15	−14/−15/−14/−16	−14/−15/−14/−17				
−15/−16/−15/−15	−15/−16/−15/−16	−15/−16/−15/−17					
−16/−17/−16/−16	−16/−17/−16/−17						
−16/−17/−16/−17							

2.2. How to perform StaRT-PCR

StaRT-PCR methods were used as described by Willey et al. (1998, 2004). First, 0.25–0.50 g fish tissue (spleen was preferred since it is readily identifiable by collectors unfamiliar with fish anatomy) was ground under liquid nitrogen using a sterile mortar and pestle, and RNA was extracted using TriREAGENT® (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's protocol. Second, RNA was re-suspended in 30 μl RNase-free water, quantified with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and the concentration was adjusted to 1 μg RNA/μl. A 30 μl RNA volume was treated with DNA-free DNase and DNAse Removal Reagents (Ambion Life Technologies, Grand Island, NY) to remove any contaminating gDNA. Third, reverse transcription of this purified RNA to cDNA was conducted using 1 μg RNA, 5X First Strand buffer, 10 mM dNTPs, 0.05 mM random hexamers, 25 U/μl RNasin, and 200 U/μl M-MLV in a 90 μl rxn volume. Reverse transcription rxns were carried out at 94 °C for 5 min, 37 °C for 1 h, and 94 °C for 5 min. All cDNA was stored at −20 °C until further use.

For each sample assessed, an initial set of PCR amplifications was conducted to determine how much cDNA and how much reference gene (*actb1*) IS should be combined to achieve approximately (within a 10-fold range) a 1:1 ratio between the NT and IS for *actb1*. For subsequent reactions, the *actb1* NT and IS concentrations were held constant for each sample, while the SMIS used (A-H) varied (Table 2) to achieve approximately (within a 10-fold range) a 1:1 ratio of the VHSv NT relative to the VHSv IS. For example, if the SMIS "D" concentration was used to assay an unknown quantity of VHSv from a fish sample, and the NT amount of VHSv was found to be >10-fold more than the VHSv IS, the next step would be to repeat the experiment using the same amount of cDNA and substituting with SMIS "C", which has a 10-fold higher concentration of VHSv IS (Willey et al., 2004).

StaRT-PCR was conducted in 10 μl rxn volumes as follows:

(A) Primer pairs for each gene were mixed together (each at 0.05 μg/μl) to decrease pipetting error, then 1 μl of the mixture for the VHSv target gene and *actb1* reference gene was placed in separate tubes.
(B) A master mixture was prepared containing both the appropriate concentration of cDNA and the appropriate SMIS (as determined in the initial set of PCR) to ensure equal loading of both into the separate rxn tubes for measurement of VHSv or *actb1*. Also included in the master mix were: Go-TAQ polymerase (final conc. of 0.1 U/μl), 10X PCR buffer containing 30 mM $MgCl_2$ (final conc. of 1X with 3 mM $MgCl_2$), dNTPs (final conc. of 0.2 mM), and RNase-free water.
(C) 9 μl of the master mixture was added into each of the tubes from (A), mixed, transferred into LightCycler® capillaries, and then PCR-amplified on a Rapid Cycler 2, as described in Section 2.1.
(D) To check for contamination in the PCR reagents and to confirm that reactions worked, a total of seven additional tubes were prepared for each experiment: (1) SMIS only (no cDNA) with primers for each gene, (2) a known VHSv positive and negative fish with primers for each gene, and (3) a water/reagent control with primers for each gene (nuclease-free H_2O).
(E) PCR products were visualized on an Agilent 2100 Bioanalyzer.
(F) This process (A-E) was repeated 3X to determine a mean and standard deviation, and calculate the standard error, for the number of VHSv molecules/10^6 *actb1* using the equations below (2 and 3).

(2) Correcting NT product size (this is necessary because quantitation was by optical density of intercalator dye and this, in turn, was related to molecule length as well as copy number).

$$\left[\frac{\text{Expected IS bp}}{\text{Expected NT bp}}\right] \times [\text{NT area under curve on Agilent graph}] = \text{Corrected NT area under curve} \quad (a)$$

$$\left[\frac{\text{Corrected NT area under curve}}{\text{IS area under curve}}\right] \times [\text{number of IS molecules in the rxn}] = \text{number of NT molecules} \quad (b)$$

(3) Normalizing VHSv to *actb1*

$$\left[\frac{\text{Number of NT molecules VHSv}}{\text{Number of NT molecules } actb1}\right] \times [10^6] = \frac{\text{VHSv molecules}}{10^6 \ actb1 \text{ molecules}}$$

2.3. Specificity, linearity, precision, and accuracy of the VHSv StaRT-PCR test

The VHSv StaRT-PCR assay was tested for non-specific amplification of several viruses (Table 3) in separate experiments. Viruses assessed included the human Encephalomyocarditis virus and Vesicular Stomatitis virus, and five fish viruses related to VHSv – Hirame Rhabdovirus, Infectious Hematopoietic Necrosis virus, Infectious Pancreatic Necrosis virus, Spring Viremia of Carp virus, and Snakehead Rhabdovirus. The latter is the sister species of VHSv and has 62% similarity (Ammayappan and Vakharia, 2009; Pierce and Stepien, 2012). Additionally, all four strains of VHSv were tested (I–IV), including 25 VHSv isolates and European, Asian, and North American variants (Table 3). Viruses were obtained either as cell culture supernatant, RNA, or tissue from infected fishes. These samples were processed following the same procedure, except that cell culture supernatants were extracted using TriREAGENT-LS® (Molecular Research Center, Inc.). All samples were assayed in triplicate.

Linearity of the StaRT-PCR assay was tested by inoculating the *Epithelioma papulosum cyprini* (EPC) cell line with known amounts of VHSv. The EPC cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were propagated

Table 3
Viral isolates screened using StaRT-PCR. –: negative result (no amplification); +: positive result.

Type	Isolate	Result
Human		
Encephalomyocarditis virus		–
Vesicular Stomatitis virus		–
Fish		
Hirame Rhabdovirus[a]		–
Infectious Hematopoietic Necrosis virus (strain 220-90)[a]		–
Infectious Pancreatic Necrosis virus		–
Snakehead Rhabdovirus[a]		–
Spring Viremia of Carp virus[a]		–
VHSv		
I	DK-F1[b]	+
Ia	FR0771[b]	+
Ia	JP96KRRV9601[b]	+
II	FI-ka663-06[c]	+
III	SM2897[d]	+
III	SC2645[d]	+
III	GH35[d]	+
III	GH 44[d]	+
IVa	Bogachiel[b]	+
IVa	Cod'91[b]	+
IVa	Elliott Bay[b]	+
IVa	JP96Obama[b]	+
IVa	Makah[b]	+
IVa	Orcas[b]	+
IVb	MI03GL[a,b]	+
IVb	vcG002[a]	+
IVb	vcG003[a]	+
IVb	vcG004[a]	+
IVb	vcG005[a]	+
IVb	vcG006[a]	+
IVb	vcG007[a]	+
IVb	vcG008[a]	+
IVb	vcG009[a]	+
IVb	vcG010[a]	+
IVc	CA-NB00-02[e]	+

[a] Isolates obtained from Western Fisheries Research Center, USGS, Seattle, WA, USA.
[b] Isolates obtained from Cornell University College of Veterinary Medicine, Ithaca, NY, USA.
[c] Isolates obtained from Finnish Food Safety Authority, Evira, Finland.
[d] Isolates obtained from Universidad de Santiago de Compostela, Spain.
[e] Isolates obtained from Fisheries and Oceans Canada, Pacific Biological Station, BC, Canada.

following Kim and Faisal (2010), trypsinized from the plate once deemed confluent, and collected as pellets after centrifugation. Pellets (three per dilution) then were spiked with a known dilution of 10^0–10^5 pfu VHSv-IVb (strain MI03GL)/10^6 cells. Two cell pellets containing nuclease-free H_2O served as negative controls. StaRT-PCR was used to measure VHSv in each sample in triplicate (totaling nine measurements per dilution). A log-log (i.e., power) regression analysis was employed to determine whether the number of molecules detected with StaRT-PCR followed a linear trend with viral dilution. Relative accuracy was quantified as the distribution of the percent difference between the number of molecules measured versus the number expected across all dilutions (Shabir, 2003). Precision, the measure of the degree of repeatability of an analytical method (Shabir, 2003), was assessed by calculating the coefficient of variation for each sample across all three experiments. Values are reported as percentages.

The true accuracy of the StaRT-PCR VHSv assay, defined as the agreement between a measurement and its known value (Shabir, 2003), was evaluated by Poisson distribution analysis (Vogelstein and Kinzler, 1999). According to this method, the laws of chance governing stochastic sampling variation were used to calculate the concentration based on the relationship between the fraction of PCRs observed to be positive relative to the fraction expected. The observations were based on nine separate PCRs each of 16 extreme limiting dilutions of a VHSv-IVb sample. VHSv-IVb was prepared from a smallmouth bass isolate of MI03GL (a homogenate of spleen, kidney, and brain tissue; provided by P. Bowser, Cornell University College of Veterinary Medicine, Ithaca, NY). MI03GL was the original IVb isolate (Elsayed et al., 2006) and is the most widespread in the Great Lakes (Thompson et al., 2011). PCRs were run on dilutions expected to contain 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 25, 50, 1.0×10^2, 2.0×10^2, 6.0×10^2, 6.0×10^3, and 6.0×10^6 VHSv molecules, which were mixed with the appropriate SMIS to achieve an approximate VHSv NT:IS ratio of 1:1. An exponential regression analysis (in SPSS v21; http://www-01.ibm.com/software/analytics/spss/; SPSS Inc., Chicago, IL; Norusis, 2008) was used to calculate the concentration of VHSv in the SMIS based on the observed: expected values. The concentration of the SMIS based on StaRT-PCR limiting dilution Poisson distribution analysis and the concentration measured by NanoDrop Spectrophotometer 2000 analysis of the undiluted stock concentration were statistically compared using a χ^2 test (in Microsoft Excel).

2.4. Effect of interfering substances on StaRT-PCR

To evaluate accuracy and performance of StaRT-PCR when subjected to possible interfering substances at the PCR level, RNA from a VHSv-IVb (MI03GL) positive smallmouth bass (also used in Section 2.3) was treated with DNA-free™ (Ambion Life Technologies, Carlsbad, CA) to remove any contaminating gDNA, and was reverse transcribed to cDNA using 1 μg RNA/90 μl rxn. The cDNA then was spiked with 0.1, 0.5, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, and 2.0 mM concentrations of EDTA per PCR and amplified in three separate StaRT-PCR runs, one series with a SMIS and the other lacking a SMIS, to assess each for the occurrence of false negatives. Products of PCR at each EDTA concentration were measured in two ways. First, the mean number of VHSv-IVb molecules/10^6 *actb1* molecules was calculated for each sample (as detailed in Section 2.2, Eqs. (2) and (3)). The number of VHSv molecules measured at 0 mM EDTA concentration was set as the 100% baseline, serving as the control. A one-way ANOVA (Sokal and Rohlf, 1995) in SPSS was used to test for significant difference in the mean number of viral molecules/10^6 *actb1* measured. Second, the total fluorescent light, measured in fluorescent light units, emitted from the Agilent intercalating dye bound to the two PCR product peaks (NT and IS) was measured on an Agilent 2100 Bioanalyzer and combined. The mean fluorescence values for each sample were calibrated as the percent change from the baseline value (0 mM EDTA control).

Reverse transcription efficiency, which is the fraction of mRNA molecules converted into corresponding cDNA molecules (Bustin and Nolan, 2004; Ståhlberg et al., 2004), was analyzed by comparing results from 1 (control), 5, 10, 20, and 30 μg of VHSv RNA/90 μl rxn. The same gDNA-free VHSv-IVb positive sample of RNA used for the EDTA test (above) was used in these experiments. A Reverse Transcription Standards Mixture (RTSM) was constructed using in vitro-transcribed RNA standards developed by the External RNA Control Consortium (ERCC; http://www.nist.gov/mml/), termed ERCC 113 and 171, and obtained from M. Salit, National Institutes of Standards and Technology, Gaithersburg, MD (Table 1). The concentration of each RNA standard was measured using a NanoDrop 2000 spectrophotometer. The ERCC 113 standard was reverse transcribed into cDNA and the cDNA was quantified using the Agilent 2100 BioAnalyzer. The ERCC 171 standard then was diluted to 1.0×10^{-10} M using 100 ng/μl yeast tRNA as a carrier, and combined with the ERCC 113 cDNA to yield a mixture of $1.59 \times 10^3 \pm 1.6 \times 10^2$ ERCC 171 RNA molecules and $1.7 \times 10^3 \pm 1.8 \times 10^2$ ERCC 113 cDNA molecules per 2 μl aliquot. A 2 μl aliquot of the RTSM was included in each of the reverse transcription rxns. The ERCC 171 RNA was converted into cDNA along with other RNA species in the reverse transcription rxn while the ERCC 113 cDNA remained unaltered.

Thus, by comparing the ratio of ERCC 171 and ERCC 113 cDNAs after reverse transcription, using StaRT-PCR, it was possible to calculate the reverse transcription efficiency. The numbers of VHSv, *actb1*, and *ef1a* molecules also were quantified in all samples using Eqs. (2) and (3) from Section 2.2. One-way ANOVAs and *t*-tests (Sokal and Rohlf, 1995) were used to identify possible differences in reverse transcription efficiency and variations in the numbers of measured molecules among the five concentrations.

2.5. Laboratory challenged fish experiments

VHSv laboratory challenge experiments were conducted at the Fish Health Laboratory Containment Facility at Michigan State University (MSU). Certified VHSv-free juvenile muskellunge were obtained from the Rathburn National Fish Hatchery (Moravia, Iowa) and maintained in covered 1900 L tanks in UV-sterilized 12 ± 1 °C oxygenated water under controlled ambient light for three weeks acclimation. Muskellunge were fed VHSv-free fathead minnows (Robinson Wholesale, Inc.) that had been determined to be VHS-free using OIE (2009) approved tissue culture isolation assays with confirmation qRT-PCR.

A subset of the 360 muskellunge (20.0 ± 10.9 g; total length 17.1 ± 1.5 cm) were randomly selected and assigned into four test groups (90 fish per group). Each group was challenged via water immersion for 90 min in 37.8 L with either: (1) a low dose of 100 pfu/ml VHSv-IVb (MI03GL), (2) a medium dose of 4.0×10^3 pfu/ml, (3) a high dose of 1.0×10^5 pfu/ml, or (4) 1 ml sterile maintenance Minimum Essential Media (MEM), which served as the negative control. The fish groups then were divided into two tank replicates (45 fish × 2), from which two fish each were selected randomly for analysis (measuring VHSv levels in their spleen tissue) at pre-determined time intervals (0, 6, 12, 24, 36 h; 2, 4, 6, 8, 15, 22, 28, 35, 42 days). Fish were euthanized immediately with an overdose of 25 mg/ml tricaine methanesulfonate (MS-222; Argent Chemical Lab), following MSU Institutional Animal Care and Use Committee approved protocols (AUF 07/07-123-00). Tanks were monitored every 8 h, and any moribund or dead fish were removed. Exterior viral particles were eliminated from the fish by submerging each 3X in double distilled H_2O. Fish were dissected under aseptic conditions with the surgical site (anus to operculum) disinfected using 100% ethanol and betadine. Spleen and head kidney were removed, placed into separate 1.5 ml tubes, flash frozen in liquid nitrogen or placed in RNAlater (Qiagen), and stored at −80 °C awaiting StaRT-PCR. Nets were disinfected using a 2% chlorhexidine solution, and sterile equipment and new gloves were used for each fish. Specimen disposal followed MSU biohazard protocols.

A second set of VHSv-IVb laboratory challenge experiments was conducted at the United States Geological Survey (USGS) Western Fisheries Research Center (WFRC) Challenge Facility in Seattle WA (under the supervision of J. Winton and F. Goetz) using six-month-old yellow perch, which were VHSv-certified-free Choptank broodstrain (Rosauer et al., 2011) from the University of Wisconsin's Great Lakes WATER Institute (Milwaukee, WI). Laboratory work was conducted under USGS-WFRC Animal Care and Use guidelines. Perch were kept in ~8 m diameter covered 278 L aquaria at 18–20 °C under ambient light conditions that mirrored the seasonal photoperiod, and fed 1.2 mm pellet feed (Oregon Biodiet, Longview, WA) every other day until satiation. A total of 210 perch were randomly selected and assigned into six groups: two groups of 38 fish (mean = 15 g) were challenged via intra-peritoneal injection of 1.0×10^5 pfu/ml VHSv-IVb (MI03GL), a group of 20 fish (mean = 15 g) was immediately euthanized, serving as a negative control, and three groups of 38 fish each (mean = ~2 g) were used for the VHSv immersion challenge, with two groups immersed for two hours in the same VHSv dosage (1.0×10^5 pfu/ml), and the final group challenged with a control dose of MEM-0 (as described above). Fish were randomly selected at pre-determined intervals (10 fish in day 1, eight in day 2, and five each for days 3–6) and euthanized using 240 mg/L MS-222 and 1.2 g/L $NaHCO_3$ following USGS-WFRC Institutional Animal Care and Use Committee protocols (2008-17). Spleen and head kidney were removed from each fish with sterile equipment, labeled, flash frozen in liquid nitrogen, and stored at −80 °C. Specimens were disposed of following University of Wisconsin's Great Lakes WATER Institute and University of Toledo biohazard protocols.

2.6. Testing for VHSv infection using StaRT-PCR and other assays

The relative performances of StaRT-PCR, conventional SYBR® green qRT-PCR, and cell culture to detect VHSv infection were compared using a χ^2 test (Sokal and Rohlf, 1995) for 23 wild-caught fishes from the Great Lakes, including: two bluegill ((*Lepomis macrochirus*), May 2011, Budd Lake, MI), a brown bullhead ((*Ameiurus nebulosus*), May 2012, Maumee Bay, Lake Erie), a freshwater drum (April 2012, Sandusky Bay, Lake Erie), seven largemouth bass (four from May 2011 and two from July 2011, Budd Lake, MI, and one from April 2012, Sandusky Bay, Lake Erie), one smallmouth bass (May 2006, Sodus Bay, Lake Ontario), and 11 lake herring ((*Coregonus artedi*), December 2009, Apostle Islands, Lake Superior). Other evaluations with StaRT-PCR included 20 experimentally challenged muskellunge (15 VHSv infected and five negative controls) and 20 of the challenged yellow perch (including seven infected fish and three negative controls from the immersion and injection challenge experiments). Cell culture was not performed on the perch samples due to the low tissue quantities available from the WFRC.

SYBR® green qRT-PCR was conducted in 25 μl rxns, containing 0.05 μg of each primer (primers were the same as those for StaRT-PCR; Table 1), 2 μl cDNA product, 10 μl SsoFast SYBR® green mix, and RNase-free water. Amplifications were carried out on a Mastercycler Realplex Thermocycler (Eppendorf, Inc., Westbury, NY) using an initial denaturation of 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, and 1 min at 60 °C. Each rxn included a known cell culture positive, a negative VHSv cDNA, and a reagent negative control (nuclease-free H_2O). Positive versus negative results for VHSv were determined based on C_t, with positives resulting in a $C_t \leq 38$. Use of that high of a C_t was facilitated by the high signal to background achieved with these optimized reagents. All samples were analyzed in triplicate, and the products were visualized on 1% agarose gels to confirm positive/negative results.

Cell culture was performed following standard OIE (2009) procedures. Spleen tissue from individual fish samples was homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd., Deans La York, UK) at high speed for 2 min and diluted with Earl's Salt-Based MEM (Invitrogen, Grand Island, NY) supplemented with 12 mM TRIS buffer (Sigma, St. Louis, MO), penicillin (100 IU/ml), streptomycin (100 μg/ml; Invitrogen), and amphotericin B (250 μg/ml; Invitrogen) to produce a 1:4 dilution of the original tissues. The dilutions were centrifuged at 2000 g and the supernatants were placed into individual wells of a 24 well plate containing confluent EPC cells, MEM, and 5% fetal bovine serum. Plates were incubated at 15 °C for seven days and observed for the formation of cytopathic effects. A second and third passage was performed before concluding infectivity.

If results were positive for cell culture (i.e., cytopathic effects were observed), RNA was extracted from infected cells following the method used for StaRT-PCR, reverse transcribed using Affinity Script Multiple Temperature Reverse Transcriptase PCR (Stratagene, La Jolla, CA), and amplified using previously described recommended standard procedures and VHSv N-gene primers (Fig. 1) (OIE, 2009).

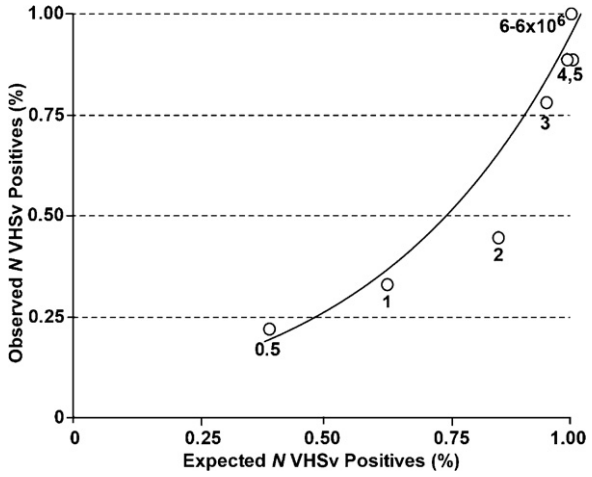

Fig. 2. Relationship between the numbers of VHSv positives discerned by StaRT-PCR (based on the % from nine separate runs of a known positive fish, for 16 dilutions of VHSv molecules) versus the number expected, as calculated via a Poisson distribution ($R^2 = 0.93$, $F = 190.10$, $df = 1, 14$, $p < 0.0001$). Dilutions were: 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 25, 50, 1.0×10^2, 2.0×10^2, 6.0×10^2, 6.0×10^3, and 6.0×10^6 VHSv molecules. Results showed that all dilutions ≥6, yielded 100% positives using StaRT-PCR (thus their ratio was 1.0 between observed and expected). Values under the points are the number of VHSv molecules measured by NanoDrop. The number of molecules detected by StaRT-PCR did not statistically differ from the NanoDrop values ($\chi^2 = 0.47$, $df = 15$, NS).

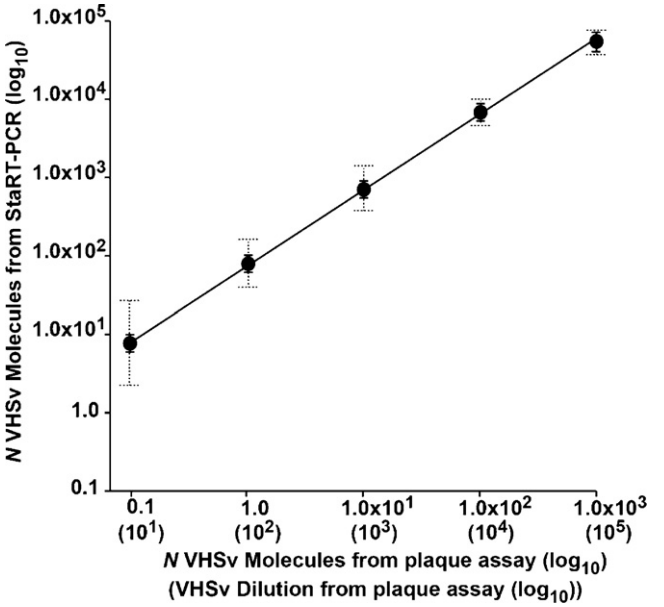

Fig. 3. The numbers of VHSv molecules determined from StaRT-PCR versus those from plaque assay (as $\log_{10}$) across a known dilution series of 10^0–10^5 pfu VHSv-IVb (strain MI03GL)/10^6 EPC cells. ●, mean of three replicate runs and three replicate measures each (nine total); SE, solid line; range, dotted line. The relationship was linear: $R^2 = 0.98$, $F = 1797.86$, $df = 1, 43$, $p < 0.001$.

2.7. Quantitative analyses using StaRT-PCR

Mean VHSv molecules calculated (from independent triplicate StaRT-PCR experiments) were compared for the wild caught and laboratory challenged fish samples. Numbers of VHSv molecules in 18 laboratory challenged muskellunge were compared between nine fish having clinical signs of infection (e.g., gross lesions and hemorrhages) and nine without signs, which were sampled at identical time points. A non-parametric Mann–Whitney U test (using SPSS) was used to rank the relative numbers of molecules in each fish. A χ^2 test (Microsoft Excel) was used to evaluate whether a threshold number of VHSv molecules characterized the appearance of clinical signs. A power analysis (G*Power2; Erdfelder et al., 1996) estimated the sample size needed for 95% confidence, using an effect size of 0.50 (Cohen, 1992). Differences in the number of VHSv molecules across days 1–6 of infection in the laboratory challenged juvenile yellow perch were tested using a one-way ANOVA. Additionally, numbers of VHSv molecules were compared between the immersion versus injected challenged individuals (60 of each).

3. Results

3.1. Performance of the VHSv StaRT-PCR test

The StaRT-PCR test was determined to be specific for VHSv diagnosis. Specifically, StaRT-PCR VHSv results were negative (no amplification) for human viruses (Encephalomyocarditis virus and Vesicular Stomatitis virus) and for other fish viruses (i.e., Hirame Rhabdovirus, Infectious Hematopoietic Necrosis virus, Infectious Pancreatic Necrosis virus, Snakehead Rhabdovirus, and Spring Viremia of Carp virus). All tests of VHSv strains and substrains (I, Ia, II, III, IVa, IVb, IVc) were positive (Table 3).

Regression analyses showed that 100% of StaRT-PCR tests at concentrations ≥6 VHSv molecules in the sample had 100% true accuracy (Fig. 2; $R^2 = 0.93$, $F = 190.10$, $df = 1, 14$, $p < 0.0001$). Experiments yielded 100% (9/9 times) amplification for dilutions of ≥6 VHSv molecules, 88% (8/9 times) for 4 and 5 molecules, 44% (4/9) for 2 molecules, 33% (3/9) for 1 molecule, and 22% (2/9) for 0.5 molecules. The number of molecules calculated from StaRT-PCR did not significantly differ from the NanoDrop measurements (Fig. 2; $\chi^2 = 0.47$, $df = 15$, NS).

StaRT-PCR quantitation of VHSv molecules in the dilution test series using biological samples followed a linear relationship to results from plaque assays, across a range of 0.1×10^0–1.0×10^3 VHSv/10^6 *actb1* molecules (Fig. 3; $R^2 = 0.98$, $F = 1797.86$, $df = 1, 43$, $p < 0.001$). The precision of the test was calculated as 9.57% for samples ≥ 11 expected VHSv/10^6 *actb1* molecules and 37.69% for ≤11 molecules.

3.2. StaRT-PCR control for interference in PCR

As measured by fluorescent light units, the amount of PCR product from positive VHSv-IVb fish RNA decreased in proportion to the

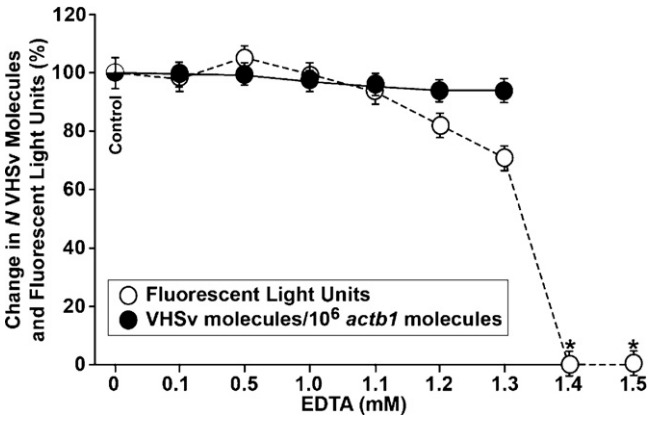

Fig. 4. The relationship between the numbers of VHSv molecules determined from StaRT-PCR versus the EDTA concentration (a test for possible inhibition). ●, mean number of VHSv molecules per 10^6 *actb1* molecules from three replicates ± SE (these changed by only 6% from 0 to 1.3 mM EDTA); ○, mean fluorescent light units from three replicates ± SE (fluorescent light units decreased, while the number of VHSv molecules remained relatively constant; $F = 4.46$, $df = 6, 14$, $p = 0.01$); *, no StaRT-PCR amplification occurred in either the NT or the IS (thus, the rxn at this point was inhibited).

amount of interfering substances in the StaRT-PCR assay (Fig. 4). For example, as EDTA increased from 0.1 to 1.3 mM, the fluorescence signal for both VHSv NT and IS PCR product decreased relative to the baseline and then disappeared at 1.4 mM and higher. However, because the number of VHSv NT molecules was measured relative to IS molecules, and because both NT and IS were affected in the same way by the presence of EDTA, the number of VHSv NT molecules measured was unaffected until the EDTA concentration increased to 1.3 mM. Even in this condition, despite the reduction of fluorescence signal to 65% of the control, measured VHSv molecules/10^6 *actb1* declined only slightly (by 6%; $F = 4.46$, $df = 6$, 14, $p = 0.01$) compared to the control. At concentrations $\geq$1.4 mM EDTA, no amplification of VHSv NT or IS occurred. Since the IS was not observed, this result would be recorded as a non-functioning assay, and not as a false negative. When testing these samples using the same protocol but without the SMIS, VHSv amplification progressively decreased with increasing EDTA concentration, with no signal at $\geq$1.4 mM EDTA. This comparison indicates that lack of inclusion of the SMIS would lead to a false negative report (indicated by *).

3.3. Optimization of reverse transcription conditions

In tests for possible RNA interference, VHSv and two reference genes, *actb1* and *ef1a*, were measured along with the RTSM across RNA concentrations from 1 (the baseline control), 5, 10, 20, and 30 μg RNA/90 μl rxn (Fig. 5). The reverse transcription efficiency, as measured by the RTSM (Fig. 5A), was significantly reduced with increasing RNA input ($F = 9.05$, $df = 4$, 10, $p = 0.002$). Differences were measured using pairwise tests between yields at 1 and 10, 20, and 30, and 10 versus 30 μg ($t = 3.10$–6.20, $df = 4$, $p = 0.003$–0.04). Overall, reverse transcription efficiency decreased 23–26% from the baseline at 20 and 30 μg RNA/90 μl rxn, respectively. Measured numbers of both reference genes increased in parallel with RNA concentration (Fig. 5B; *actb1*: $F = 96.45$, $df = 4$, 10, $p < 0.001$; *ef1a*: $F = 27.36$, $df = 4$, 10, $p < 0001$), yet remained constant when ef1a was normalized to *actb1* ($F = 1.01$, $df = 4$, 10, NS). Measured numbers of VHS molecules, in contrast to what we predicted, increased significantly with increasing RNA input from 1 to 10 μg (Fig. 5C; $t = 2.82-3.46$, $df = 4$, $p = 0.03-0.05$), but showed no further increase with additional RNA/reverse transcription ($t = 0.32-1.68$, $df = 4$, NS). The number of VHSv molecules per 10^6 *actb1* molecules significantly decreased with addition of RNA/reverse transcription ($F = 282.64$, $df = 4$, 10, $p < 0.001$).

3.4. VHSv detection and quantitation in wild caught and laboratory challenged fishes

StaRT-PCR had greater accuracy than conventional SYBR® green qRT-PCR, with the latter having 40% false negative error in experiments with wild caught fishes (Fig. 6A; 10 vs. 6 positives; $\chi^2 = 1.53$, $df = 1$, NS), 47% error for immersion challenge muskellunge (Fig. 6B; 15 vs. 8; $\chi^2 = 5.01$, $df = 1$, $p = 0.03$), and 14% error for challenged yellow perch (Fig. 6C; 14 vs. 12; $\chi^2 = 0.44$, $df = 1$, NS). StaRT-PCR also

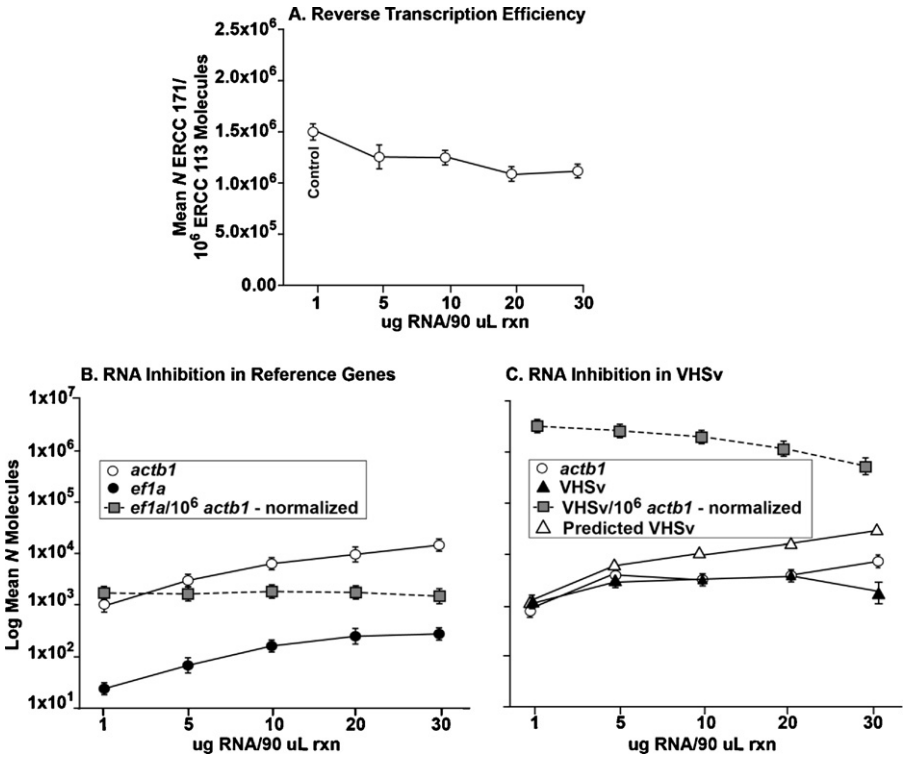

Fig. 5. Mean reverse transcription efficiency and performance of StaRT-PCR under possible RNA inhibition conditions (± SE from three replicates). (A) The relationship between the mean numbers of ERCC 171 molecules/10^6 ERCC 113 molecules from StaRT-PCR versus the concentration of VHSv positive RNA used in the rxn (μg RNA/90 μl). Reverse transcription efficiency significantly changed from 1 (the baseline control), 5, 10, 20, and 30 μg RNA/90 μl rxn ($F = 9.05$, $df = 4$, 10, $p = 0.002$), with significant pairwise tests at 1 and 10, 20, and 30, and 10 versus 30 μg ($t = 3.10$–6.20, $df = 4$, $p = 0.003$–0.04). (B) The relationship between the number of molecules from StaRT-PCR using the reference genes *actb1* and *ef1a* versus the concentration of RNA used in the rxn (μg RNA/90 μL). The numbers of *actb1* and *ef1a* molecules both increased with RNA concentration (*actb1*: $F = 96.45$, $df = 4$, 10, $p < 0.001$; *ef1a*: $F = 27.36$, $df = 4$, 10, $p < 0.001$), but remained relatively constant (dotted line) when *ef1a* was normalized to *actb1* (reported as $\log_{10}$; $F = 1.01$, $df = 4$, 10, NS). (C) The relationship between the mean number of molecules from StaRT-PCR, using the target gene VHSv and the reference gene *actb1*, versus the concentration of RNA used in the rxn (μg RNA/90 μL). In contrast to predicted values, VHS molecules increased significantly from 1–10 μg ($t = 2.82-3.46$, $df = 4$, $p = 0.03-0.05$), but not with any further increase in RNA ($t = 0.32-1.68$, $df = 4$, NS). The number of VHSv molecules per 10^6 *actb1* molecules significantly decreased (dotted line) as more RNA/reverse transcription was added (reported as $\log_{10}$; $F = 282.64$, $df = 4$, 10, $p < 0.001$).

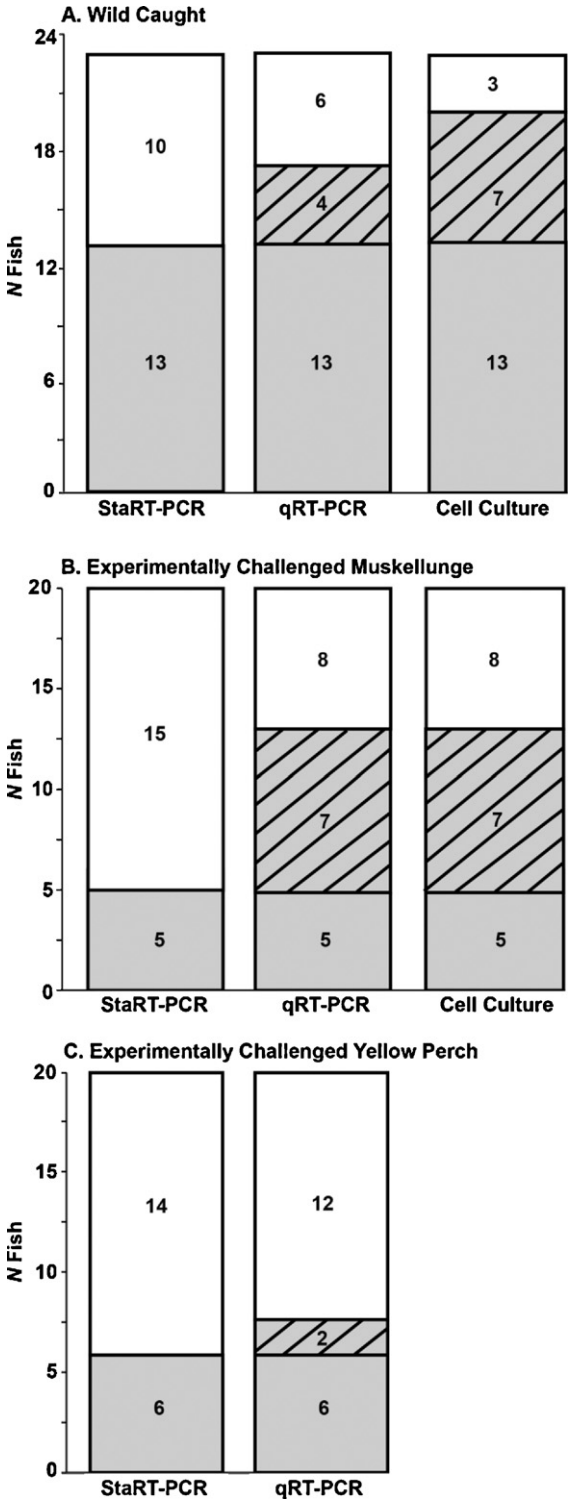

Fig. 6. Comparisons among assay test results for VHSv infection from StaRT-PCR, SYBR® green qRT-PCR, and cell culture for (A) Wild caught fishes, (B) Muskellunge challenge experiments, and (C) Yellow perch challenge experiments (cell culture not available). Gray = negative test result, gray with hash marks = false negative, white = positive test result. Compared with StaRT-PCR, for (A) SYBR® green qRT-PCR had 40% false negative error ($\chi^2 = 1.53$, $df = 1$, NS) and cell culture had 70% error ($\chi^2 = 10.39$, $df = 1$, $p = 0.001$). For (B), SYBR® green qRT-PCR and cell culture both had 47% false negative error ($\chi^2 = 5.01$, $df = 1$, $p = 0.03$). For (C), SYBR® green qRT-PCR had 14% false negative error ($\chi^2 = 0.44$, $df = 1$, NS).

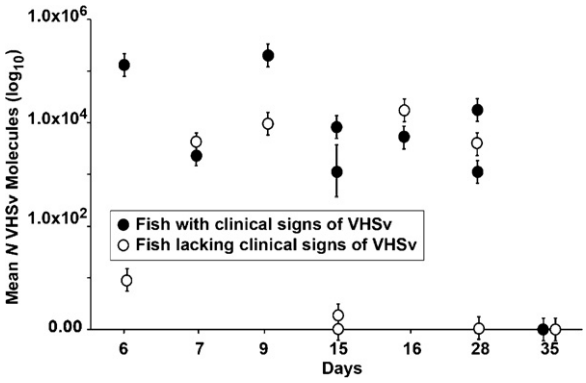

Fig. 7. Comparative numbers of VHSv molecules per 10^6 *actb1* molecules ($\log_{10}$ mean ± SE of three replicate runs) in laboratory challenged muskellunge with (●) and without (○) clinical signs of VHSv infection, measured using StaRT-PCR. Values for the two groups statistically differed in a Mann Whitney ranking test ($Z = -2.10$, $U(df) = 1$, $p = 0.04$ for days 6–28).

detected significantly more VHSv positives than did cell culture, with the latter having 70% false negative error for wild caught fishes (Fig. 6A; 10 vs. 3; $\chi^2 = 10.39$, $df = 1$, $p = 0.001$), and 47% error in laboratory challenged muskellunge (Fig. 6B; 15 vs. 8; $\chi^2 = 5.01$, $df = 1$, $p = 0.03$).

SYBR® green qRT-PCR correctly detected slightly more positives than did cell culture in the wild caught fishes (Fig. 6A; 6 vs. 3, 50% error difference; $\chi^2 = 1.24$, $df = 1$, NS); both diagnosed equivalent numbers of positives in the experimentally challenged muskellunge (Fig. 6B; 8 vs. 8; $\chi^2 = 0.00$, $df = 1$, NS). All positives detected by cell culture and SYBR® green qRT-PCR were also positive with StaRT-PCR. The false negative range for SYBR® green qRT-PCR was 1.0×10^0–6.5×10^1 VHSv molecules (as quantified by StaRT-PCR) and 1.0×10^0–1.0×10^3 molecules for cell culture. For all assays, negative controls yielded negative results; i.e., they had no false positives and no contamination.

Numbers of VHSv molecules measured by StaRT-PCR varied widely among wild caught specimens (1.0×10^0–1.2×10^5 VHSv molecules/10^6 *actb1* molecules) and laboratory challenged individuals (1.0×10^0–8.4×10^5/10^6 *actb1* molecules). Fig. 7 shows a difference in the mean numbers of VHSv molecules between muskellunge exhibiting clinical signs of infection ($1.9 \times 10^4 \pm 1.2 \times 10^4$) versus those without ($1.1 \times 10^3 \pm 4.5 \times 10^2$; $Z = -2.10$, $U(df) = 1$, $p = 0.04$, days 6–28). At day 35, no VHSv was detected in remaining fish from either group. Numbers of VHSv molecules appeared to differ at days 6, 9, 15, and 28, with those showing clinical signs of infection having higher values (Fig. 7). One hundred VHSv molecules were estimated to distinguish a threshold at which individuals showed clinical signs of infection ($\chi^2 = 0.09$, $df = 1$, NS). Power calculations determined that 52 fish samples (26 with and 26 without clinical signs) would be needed to verify this level (95% confidence interval) in further testing.

Quantities of VHSv measured in the yellow perch laboratory challenge experiments ranged from 4.0×10^0 to 1.3×10^5 VHSv molecules/10^6 *actb1* molecules in the immersion challenged fish and 1.0×10^0–1.8×10^5 molecules in the injection challenged individuals. Results of the yellow perch laboratory challenge experiments showed that VHSv infection first was detected at day three in immersion challenged fish, versus day two in injection challenged individuals (Fig. 8; $t = 2.15$, $df = 19$, $p = 0.04$). Overall numbers of VHSv molecules appeared relatively consistent across the remaining course of infection through day six (immersion: $F = 0.48$, $df = 3, 29$, NS; injection: $F = 2.62$, $df = 4, 42$, $p = 0.05$), and did not differ between the two experiments ($t = 0.03$–0.96, $df = 13$–18, NS).

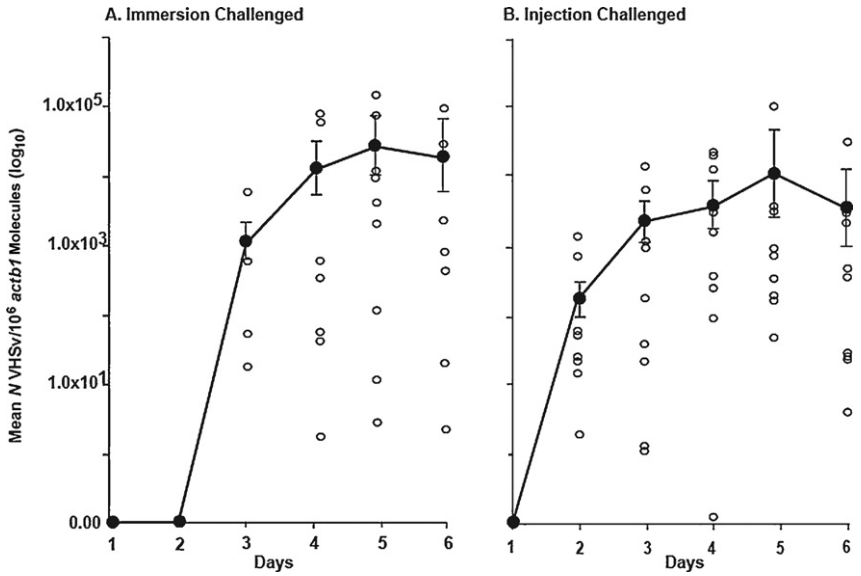

Fig. 8. Numbers of VHSv molecules per 10^6 *actb1* molecules measured by StaRT-PCR (mean ± SE from three replicates; reported as $\log_{10}$) across the early stages of infection in yellow perch for (A) Injection challenge and (B) Immersion challenge experiments. ●:overall mean. ○:individual measures. The experiments differed in their onsets of infection, with (A) being three days and (B) being two ($t = 2.15$, $df = 19$, $p = 0.04$). Overall numbers of VHSv molecules remained consistent across the infection course (A: days 3–6, $F = 0.48$, $df = 3, 29$, NS; B: days 2–6, $F = 2.62$, $df = 4, 42$, $p = 0.05$). There was no difference in the number of VHSv molecules between A and B ($t = 0.03$–0.96, $df = 13$–18, NS).

4. Discussion

4.1. Specificity and accuracy of the StaRT-PCR VHSv test (Hypotheses 1 and 2)

The fisheries, aquaculture, and baitfish industries rely on accurate certification of their products as VHSv-free (Aquatic Invasive Species Action Plan, 2011), for which results from cell culture may take up to 28 days (Garver et al., 2011) and frequently yield false negative conclusions (47–70% reported in this study). Thus, the development of a rapid and accurate diagnostic assay is key to preventing VHSv spread to new areas, species, and populations (Cutrín et al., 2009). Similar to other PCR-based assays developed to detect VHSv, the StaRT-PCR test is VHSv specific, supporting hypothesis H_A1. StaRT-PCR detected all VHSv strains and substrains, and did not amplify other viruses.

Antibody assays (Millard and Faisal, 2012) and other PCR tests (Chico et al., 2006; López-Vázquez et al., 2006; Liu et al., 2008; Matejusova et al., 2008; Cutrín et al., 2009; Hope et al., 2010; Garver et al., 2011; Jonstrup et al., 2013; Phelps et al., 2012), lacked internal controls. Those other PCR tests often resulted in false negatives, leading to inaccurate conclusions, as demonstrated by the present investigation's SYBR® green qRT-PCR results (14–47% false negative error).

4.2. Quantitation of VHSv by StaRT-PCR (Hypothesis 2)

Results support hypothesis H_A2 that StaRT-PCR accurately quantifies VHSv and is effective across all levels of the virus tested, from low to high. Other assay methods were unable to discern lower levels of virus, resulting in false negatives. Notably, cell culture was unable to discern levels from 1.0×10^0 to 1.0×10^3 VHSv/10^6 *actb1* molecules and SYBR® green qRT-PCR failed to detect from 1.0×10^0 to 6.5×10^1 molecules. Fish harboring low levels of the virus thus would pass inspection for VHSv-free certification, using those assays.

PCR-based methods have been shown to be more sensitive than traditional cell culture (e.g., Chico et al., 2006; López-Vázquez et al., 2006; Hope et al., 2010; Garver et al., 2011; Jonstrup et al., 2013) since they amplify replicating (i.e., infectious) and non-replicating (i.e., non-active) transcripts alike. Cell culture solely detects actively replicating virus that is capable of infecting cells. Thus, it was anticipated that StaRT-PCR, like other RT-PCR approaches, would yield more positives than cell culture. However, detection of both replicating and non-replicating VHSv RNA could be advantageous since its presence may indicate that there are active infections in the area where the samples were collected. This information may aid management efforts and aquaculture facilities to identify latent infections that could be negative by cell culture, but potentially infective.

StaRT-PCR detected more positives than SYBR® green qRT-PCR, which was not surprising as it was more thoroughly optimized for PCR efficiency and controlled for false negatives. Compared to other PCR tests, StaRT-PCR detected a lower threshold of molecules. For example, Garver et al. (2011) stated that their two-step qRT-PCR assay diagnosed 100 VHSv N-gene copies with 100% accuracy. In contrast, StaRT-PCR showed 100% accuracy for six molecules and detected samples of a single molecule at a frequency limited only by the laws of chance governing stochastic sampling variation.

4.3. Performance in presence of PCR inhibitors (Hypothesis 3)

StaRT-PCR effectively controlled for inhibition at the PCR level, supporting H_A3. In contrast, Hope et al.'s (2010) one-step qRT-PCR test showed that increasing the VHSv concentration in reverse transcription rxns from 50 ng to 1 μg RNA/25 μL resulted in only a 10-fold increase in PCR product, two-fold less than what they expected, which could lead to false negatives. They observed even more dramatic reduction in reverse transcription efficiency with higher RNA input. Based on our results, we conclude that 1 μg/90 μl reverse transcription rxn will provide a test with the best balance between optimal sensitivity and adverse effects of RNA input on reverse transcription efficiency. Further increase in RNA input to 10 μg/90 μl will yield further significant increase in VHSv amplification, but the gain will be marginal relative to the RNA consumed due to decreasing reverse transcription efficiency.

Degraded or environmentally challenged samples can lead to loss of signal and interfere with detection of the virus (see McCord et al., 2011). Such samples often contain substances that are co-extracted with the RNA or carried over into the cDNA via the reverse

transcription rxn, which may inhibit or lower PCR efficiency by binding to the polymerase and/or blocking reagents necessary for amplification (Opel et al., 2010). StaRT-PCR is the sole VHSv diagnostic to test, detect, and control for PCR inhibition, avoiding false negative results when samples contained inhibitors such as EDTA.

The amount of VHSv PCR product decreased as the reverse transcription efficiency decreased, in contrast to the increase in product yield from the two reference genes, indicating that detection of this virus may be affected by RNA interference differently than the endogenous controls. The difference between them might be due to contaminating VHSv protein features that are tightly bound and not removed during the RNA extraction procedure. Antigenic sites or secondary structures (α-helicase, β-strands and loops), which are common in *Novirhabdoviruses* (Walker and Kongsuwan, 1999), may contribute to this difference. Other unknown factors may be responsible, which merit further investigation. To avoid these issues, a standard RNA input/reverse transcription condition should be employed when assaying for VHSv, for which 1 μg RNA/90 μl rxn is recommended.

4.4. StaRT-PCR performance versus other assays (Hypotheses 4 and 5)

StaRT-PCR results diagnosed and quantified the amount of VHSv in fish samples from the field and laboratory experiments, supporting H_A4. Like most other PCR-based VHSv detection methods (Chico et al., 2006; López-Vázquez et al., 2006; Hope et al., 2010), StaRT-PCR is more sensitive and accurate than the traditional "gold-standard" cell culture approach in detecting qualitative positives. In contrast to the present findings, Jonstrup et al. (2013) found that cell culture significantly exceeded the detection ability of their one-step qRT-PCR test at low titer (1.9×10^2–1.9×10^3 $TCID_{50}$/ml). Thus, their test, unlike StaRT-PCR, did not outperform cell culture.

Besides the present investigation, only two other studies compared detection levels of different PCR assays. López-Vázquez et al. (2006) found that their two-step nested qRT-PCR assay had 15–80% false negatives, their qualitative one-step qRT-PCR assay had 60–90%, and cell culture had 95%. Chico et al. (2006) reported that their two-step TAQman® qRT-PCR test had an error rate of 25% (detected 9 of 12 positives) versus 92% error in two-step nested qRT-PCR assays (found 1 of 12 positives), and 67% error with cell culture (identified 4 of 12 positives). In contrast, StaRT-PCR in the present study yielded 0 false negatives, versus 14–47% false negatives with two-step SYBR® green qRT-PCR, and 47–70% with cell culture. The error rates found with SYBR® green qRT-PCR and cell culture in the present study thus were similar to those reported by Chico et al. (2006).

Most other PCR methods conducted a dilution series with a regression analysis to evaluate sensitivity (e.g., Chico et al., 2006; López-Vázquez et al., 2006; Matejusova et al., 2008; Cutrín et al., 2009; Hope et al., 2010; Garver et al., 2011; Jonstrup et al., 2013; Phelps et al., 2012). This also was done in the present study, along with an extreme limiting dilution assay that compared the observed frequency of positive StaRT-PCR assays against those predicted by Poisson distribution. Thus, the current investigation evaluated the absolute accuracy of test results, incorporating stochastic sampling variation. The close agreement between the values measured with spectrophotometric quantitation and those expected from the Poisson distribution supports the reliability of StaRT-PCR quantitation capability (Vogelstein and Kinzler, 1999). Based on this analysis, the StaRT-PCR assay measures to a single VHSv molecule with known true accuracy, unlike other PCR methods (Liu et al., 2008; Hope et al., 2010; Garver et al., 2011), whose minimum detection thresholds were much higher (requiring ≥100 copies of VHSv). Thus, StaRT-PCR has greater sensitivity and accuracy than cell culture or other qRT-PCR tests, supporting H_A5.

4.5. Biological levels of VHSv detected with StaRT-PCR (Hypotheses 6–8)

The application of employing StaRT-PCR to quantify VHSv infection was demonstrated in a variety of biological experiments. Results show that levels of VHSv molecules in wild caught fishes vary widely (1.0×10^0–1.2×10^5 VHSv/10^6 *actb1* molecules), resembling values measured in laboratory challenged individuals (1.0×10^0–8.4×10^5). Additionally, mean VHSv levels in laboratory challenged individuals showing signs of VHSv infection were higher than without, supporting H_A6. This suggests that individuals in a population respond variably to VHSv infection, underscoring the importance of an improved diagnostic test for managing this disease. A threshold level of 100 VHSv molecules was identified as a potential biomarker for clinical signs of infection. This observation supports the conclusion that the greater sensitivity of StaRT-PCR compared to cell culture or SYBR® green qRT-PCR has biological significance. Further experiments are warranted to validate this finding.

To the authors' knowledge, only a single other investigation quantified VHSv samples from infected fish samples. Similar to StaRT-PCR results, Chico et al. (2006) determined that VHS viral load varied widely among nine rainbow trout individuals in an immersion challenge (measured as VHSv relative to RNA). Chico et al. (2006) was one of four other studies that have used a reference gene (*18srRNA*) for quantitation. Matejusova et al. (2008), Garver et al. (2011), and Jonstrup et al. (2013) employed a different reference gene (*ef1a*) to confirm RNA quality for cDNA synthesis and to serve as a positive control, but did not quantify amounts of VHSv. The present study evaluated both of those reference genes (*18srRNA* and *ef1a*), as well as *actb1*, facilitating accurate quantitation of VHSv.

Over recent years, increasing numbers of individuals and species of Great Lakes fishes have tested positive for VHSv-IVb, yet many of those have appeared healthy (Kim and Faisal, 2010). For example, a freshwater drum collected in the Lake Erie Harbor of Sandusky Bay on April 12, 2012 and a largemouth bass on May 10, 2012 that tested positive for VHSv with StaRT-PCR (3.4×10^2 and 5.9×10^2 VHSv/10^6 *actb1* molecules, respectively), did not have lesions, but were swimming erratically. Lack of external VHS hemorrhages renders it difficult to determine whether fish have VHS and potentially could transmit the virus. As described above, our results in experimentally challenged fish suggest that a measured value of greater than 100 using StaRT-PCR may be associated with clinical signs of infection.

No other study to date has monitored levels of VHSv molecules across the early course of infection. The relative number of VHSv molecules was tracked in experimentally challenged yellow perch from days one to six. Onset of VHSv infection occurred a day later in immersion challenged than in injection challenged fish, with their viral concentrations remaining relatively consistent through day six, supporting H_O7. Quantities of virus did not differ between the immersion versus injection challenged fish after onset of infection (H_O8). Both experiments showed a linear increase in the number of VHSv molecules from the onset of infection, followed by a plateau phase, and a decrease at day six. These data may indicate that VHSv levels are highest at the beginning of the infectious stage, level off as the host develops its immune response, and then start to decline. However, these results are based solely on early infection, so future studies that include middle and later stages are needed to better understand VHSv infection patterns, the disease course, and host response.

4.6. Summary and conclusions

The cell culture diagnostic approved for VHSv-free certification (Aquatic Invasive Species Action Plan, 2011) is lengthy, labor intensive, and lacks sensitivity compared to PCR-based assays. Reliance on cell culture alone could result in spread of VHSv throughout aquaculture systems, baitfish transport, and/or watersheds, leading to significant losses. The other PCR-based techniques to detect VHSv (Chico et al., 2006; López-Vázquez et al., 2006; Liu et al., 2008; Matejusova et al., 2008; Cutrín et al., 2009; Hope et al., 2010; Garver et al., 2011; Jonstrup et al., 2013; Phelps et al., 2012) lacked the intrinsic quality control necessary for accurate and reliable detection. Moreover, those tests were unable to measure their accuracy and reverse transcription efficiency. The new StaRT-PCR assay incorporates IS that allow for sensitive and accurate qualitative and quantitative measurements of VHSv. These standards guard against false negative results and correct for interfering substances. In addition, the present investigation uses StaRT-PCR to demonstrate reverse transcription efficiency and determine a biological threshold level for clinical signs of infection. Implementation of the StaRT-PCR test will aid aquaculture, baitfish, and fishery industries via faster, more sensitive, and accurate disease detection. Application may lead to improved management and commerce, and lead to cost-savings for stakeholders. This StaRT-PCR diagnostic will enhance natural resource conservation efforts to detect the virus and track its spread.

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