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ARTICLE

The Use of a One-Step Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) for the Surveillance of Viral Hemorrhagic Septicemia Virus (VHSV) in Minnesota

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Abstract

Viral hemorrhagic septicemia virus (VHSV) is a highly contagious and pathogenic virus of fish. The virus infects more than 70 fish species worldwide, in both fresh and salt water. A new viral strain (VHSV-IVb) has proven both virulent and persistent, spreading throughout the Great Lakes of North America and to inland water bodies in the region. To better understand the geographic distribution of the virus, we used a modified real-time reverse transcription polymerase chain reaction (rRT-PCR) assay for high-throughput testing of fish for VHSV. The assay was shown to be twice as sensitive as the gold standard, virus isolation, and did not cross react with other viruses found in fish. In addition, the diagnostic turnaround time was reduced from 28 to 30 d for virus isolation to 2–4 d for rRT-PCR. To demonstrate the usefulness of the rRT-PCR assay, 115 high-priority water bodies in Minnesota were tested by both methods from April 2010 to June 2011. All survey sites tested negative for VHSV by both methods. The survey results have informed fisheries managers on the absence of VHSV in Minnesota and have better prepared them for the eventual arrival of the disease. In addition, the results demonstrate the value of this rRT-PCR as a surveillance tool to rapidly identify an outbreak so that it can be controlled in a timely manner.

Viral hemorrhagic septicemia virus (VHSV) is a highly contagious and pathogenic virus of fish. The virus has a broad host range including more than 70 fish species and is capable of causing significant biological and economic losses (OIE 2009; Kim and Faisal 2011). The presence of this virus was first suspected as early as 1938, but was not confirmed until 1963 when VHSV was isolated from freshwater rainbow trout *Oncorhynchus mykiss* from farms in Denmark (Schaperclaus 1938; Jensen 1963). Currently, there are four genotypes of VHSV (VHSV-I to VHSV-IV). Genetic analysis of viral isolates through the 1990s from Europe (VHSV-I, II, III), East Asia (VHSV-I, II, IV), and North America (VHSV-IV) concluded that VHSV is of marine origin but has also mutated to cause disease in freshwater fish hosts (Einer-Jensen et al. 2004; Pierce and Stepien 2012). The routes of transmission between regions and environments are not fully known, but the transfer of infected water, baitfish, and fish for stock enhancement are likely candidates (Herve-Claude et al. 2008; Bain et al. 2010; VHSV Expert Panel and Working Group 2010).

In 2003, VHSV was isolated in the Laurentian Great Lakes (Lake St. Clair) basin from a muskellunge *Esox masquinongy*. This was a first event in several regards: (1) presence of VHSV in freshwater of North America, (2) susceptibility of this particular host species, and (3) presence of fish rhabdovirus in the Great Lakes basin (Kim and Faisal 2011). Unfortunately, this isolate was not confirmed to be VHSV until 2005 during a second muskellunge mortality event (Elsayed et al. 2006). Since then the virus has proven to be highly pathogenic in this new environment, naturally infecting 28 different fish species and causing significant losses throughout much of the Great Lakes basin (USDA-APHIS 2010; Kim and Faisal 2011; Thompson et al. 2011). Although confirmed positive for VHSV by surveys of apparently healthy fish, Lake Superior has not experienced mass mortality events similar to those in

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the eastern Great Lakes (Bain et al. 2010; Thompson et al. 2011).

Within the North American VHSV genetic group IV, the marine and freshwater isolates are grouped into VHSV-IVa and VHSV–IVb, respectively (Meyers and Winton 1995). The genetic divergence within VHSV-IVb is very low (maximum of 1.05% in 669 nucleotides of the G-gene), which is consistent with a recent introduction of the virus in a naive population (Thompson et al. 2011; Pierce and Stepien 2012). Although the differences were minor, Thompson et al. (2011) did identify multiple unique isolates in single outbreak locations, including inland lakes. This has supported the covert nature of the virus and its ability to repeatedly move or be introduced undetected. Therefore, continued virus surveillance is critically important to reduce further spread of VHSV within, and out of, the Great Lakes basin.

Several methods are currently utilized for regulatory, clinical, and surveillance testing of VHSV. Virus isolation is the gold standard diagnostic assay for all fish viruses as recommended in the U.S. Fish and Wildlife Service (USFWS) and American Fisheries Society, Fish Health Section (AFS-FHS) Blue Book (USFWS and AFS-FHS 2010) and the World Organization for Animal Health Manual of Diagnostic Tests for Aquatic Animals (OIE 2009). As such, virus isolation is widely used and accepted for regulatory testing of fish to certify virus-free status (Purcell et al. 2011). However, for surveillance or other voluntary testing without movement or legal implications, the selection of diagnostic tests can be much more flexible. While an assay for surveillance purposes must be validated and proven robust to high standards, it should be best fit for the purpose. One method that has gained popularity in recent years for surveillance testing is real-time polymerase chain reaction (rt-PCR) (Mackay et al. 2002; Walker 2002; Espy et al. 2006). The rt-PCR can improve sensitivity, speed, and accuracy, while lowering cost, compared with traditional methods of virus isolation (Leland and Ginocchio 2007; Hope et al. 2010). These advantages allow managers to have a better understanding of virus distribution and respond more rapidly to an outbreak. Limitations, such as poor validation and risk of cross-contamination in molecular assays, have been significantly reduced with increases in laboratory experience and technological advancements (OIE 2009; Purcell et al. 2011).

Real-time PCR has been widely used and federally recognized as a primary detection and surveillance assay for a variety of terrestrial animal diseases including avian influenza, exotic Newcastle disease, classical swine fever, swine influenza, foot and mouth disease, and many others (OIE 2009; USDA-APHIS 2010; USFDA 2011). While the same cannot be said for rt-PCR for aquatic pathogens, there has been widespread development and validation for their use in research and clinical cases (Purcell et al. 2011). Both conventional reverse transcription PCR (RT-PCR) and real-time RT-PCR (rRT-PCR) methods exist for the detection of VHSV in fish (Winton and Einer-Jensen 2002; Hope et al. 2010; Garver et al. 2011; Jonstrup et al., in press). These assays have been validated to varying standards and have been designed for specific purposes. For example, conventional RT-PCR is more commonly used for confirmatory testing following virus isolation (OIE 2009; USFWS and AFS-FHS 2010). Two of the previously published rRT-PCR assays were designed to detect all known strains of VHSV (Garver et al. 2011; Jonstrup et al., in press), while another assay was designed to detect only the VHSV-IVb strain in the Great Lakes (Hope et al. 2010). All of these assays have demonstrated their value as a surveillance tool by increasing sensitivity and significantly reducing turnaround time compared with virus isolation (Knuesel et al. 2007; Bain et al. 2010; Hope et al. 2010; Garver et al. 2011; Jonstrup et al., in press).

Until the regulatory framework is established, the voluntary use of rRT-PCR for the detection of VHSV can significantly improve management of this virus. For example, prior to 2010, the majority of Minnesota water bodies used for aquaculture or recreational purposes were never tested for VHSV. As a result, the VHSV status for much of the state was unknown, despite routine movements of fish between water bodies for bait and stock enhancement. In the present study, a one-step version of the Garver et al. (2011) assay was used for high-throughput testing of fish for VHSV surveillance. This initial survey has informed epidemiologists and managers on the extent of the virus presence in Minnesota, which has better prepared them for the eventual arrival of VHSV. In addition, the survey has satisfied the necessary regulatory testing requirements for aquaculture producers to move fish within the state (Minnesota statutes 2011, Section 17.4991, subdivision 3).

METHODS

Source of samples.—A survey of Minnesota fish populations was performed for the presence of VHSV from April 2010 through June 2011. Registered license holders for aquaculture facilities ($n = 145$) and baitfish harvesters ($n = 289$) were contacted by mail to participate in the surveillance program. The program offered a free "regulatory inspection," which consisted of collecting fish and testing them simultaneously by virus isolation (the gold standard test) and modified rRT-PCR. Fish species included in the survey were limited to those on the U.S. Department of Agriculture, Animal and Plant Health Inspection Service VHSV susceptible species list (USDA-APHIS 2008) at the time of the survey. Sample locations were selected based on (1) water body use by the aquaculture industry, (2) presence of VHSV susceptible species, (3) distribution throughout Minnesota, and (4) high risk or high priority as identified by the Minnesota Department of Natural Resources (MNDNR).

On-site fish collection was overseen by an accredited veterinarian or MNDNR field biologist with specialized fish health training, or by an American Fisheries Society certified Fish Health Inspector. A minimum of 60 fish per site were collected, targeting species on the aquaculture permit, such as the spottail shiner *Notropis hudsonius* and walleye *Sander vitreus*. If the target species was unattainable owing to seasonal

availability, 150 fish of other VHSV susceptible species were collected. For large populations (>1,000 fish), sampling of 60 fish provided 95% confidence in detecting VHSV, assuming the virus was present in 5% of the population. The sample of 150 susceptible fish maintained 95% confidence, but accounted for a potentially lower prevalence of 2%. Fish collections primarily occurred during the spring and fall when water temperatures were suitable for VHSV.

Freshly dead fish were submitted on ice to the Minnesota Veterinary Diagnostic Laboratory (MVDL) within 24 h of collection. Immediately upon arrival, each fish was visually inspected for clinical signs of disease and a necropsy was performed to obtain the appropriate samples e.g., kidney and spleen from fish >6.0 cm, entire viscera from fish 4.0 to 6.0 cm, and the entire fish when it was <4.0 cm in length. Tissues from five fish were pooled together and stored at 4°C for no more than 24 h. The pools were tested for VHSV by both virus isolation and rRT-PCR. Results were made immediately available online for the owner and the regulatory agency for review.

Sample preparation.—Because of regulatory implications of the survey results, all samples from this study were tested by both virus isolation and rRT- PCR. Tissue processing was identical for both methods and followed the protocols recommended by USFWS and AFS-FHS Blue Book (2010). Briefly, a 10% suspension of the tissue was prepared in Hanks' balanced salt solution (HBSS). The suspension was processed in a stomacher for 30 s followed by centrifugation at 4°C for 15 min at 2,900 × *g*. An equal amount of antibiotic incubation medium was added to the supernatant. After mixing, the suspension was incubated for 2 h at 15°C and finally recentrifuged for 15 min at 2,900 × *g*. The resulting supernatant was immediately used for virus isolation and rRT-PCR.

Virus isolation.—Virus isolation was performed according to the USFWS and AFS-FHS Blue Book (2010). Monolayers of epithelioma papulosum cyprini (EPC) cells (Fijan et al. 1983) were prepared in 48-well microtiter plates. When monolayers were 80% confluent, the cell culture media was decanted and the sample was inoculated (100 μL/well). The inoculated cells were incubated at 15°C for 60 min to allow for viral adsorption. Maintenance medium was then added and the plates incubated at 15°C for 14 d. The plates were examined twice weekly for the appearance of cytopathic effects (CPE). Unless CPE was observed, all samples were blind passaged at day 14 and determined negative if no CPE appeared after 14 d of incubation. A VHSV-IVb isolate (Great Lakes reference strain: MI03) was used as a positive control to monitor the sensitivity of the cell line to the virus. The CPE from the positive control was occasionally confirmed by rRT-PCR, sequencing, and negative contrast electron microscopy. Cell culture supernatant was stored at 4°C for no more than 24 h prior to extraction.

Total nucleic acid extraction.—MagMAX Express 96 Viral RNA Isolate kit (AM1836; Life Technologies, Grand Island, New York) was used for total nucleic acid extraction from sample suspension and from infected cell culture supernatants. The extraction was performed according to a custom protocol developed by the MVDL in association with Life Technologies. The primary difference from the standard protocol is a starting volume of 100 μL and final elution volumes of 75 μL, compared with 50 μL for each in the standard protocol. This method was originally optimized for high-throughput extraction of a swine virus, the porcine reproductive and respiratory syndrome virus (PRRSV), and has been found to be effective for a wide variety of other animal pathogens (data not shown). Along with unknown samples, a VHSV-IVb positive isolate grown in EPC cells was extracted for each run to serve as a positive extraction control. The extraction procedure was also performed on 100 μL of 1 × PBS to serve as a negative extraction control. The resulting RNA was stored on ice for immediate testing or frozen at −80°C for later testing.

Real-time reverse transcription polymerase chain reaction.—Real time RT-PCR for VHSV was performed on an ABI 7500 Sequence Detection System (Life Technologies). The master mix was prepared in a 20-μL volume, with 12.5 μL of 2× Path-ID Multiplex RT-PCR Buffer (Life Technologies), 1.25 μL of 10× Path-ID Multiplex enzyme mix (Life Technologies), 1.0 μL of 5 μM FAM dye-labeled MGB Probe (5′-Fam-TAC GCC ATC ATG ATG AGT-3′), 0.375 μL of each 40-μM forward (5′-ATG AGG CAG GTG TCG GAG G-3′) and reverse (5′-TGT AGT AGG ACT CTC CCA GCA TCC-3′) primers, and 4.5 μL of nuclease-free water. The primer–probe set was designed to detect the N-gene of all known strains of VHSV (Garver et al. 2011). Five microliters of extracted RNA (unknown samples), nuclease-free water (negative control), VHSV-known-positive RNA (positive PCR control), VHSV-known-positive cell culture supernatant (positive extraction control), or negative extraction control were added to each well. Each sample was run in duplicate. The thermal cycling protocol consisted of 10 min at 45°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

Analytical specificity and sensitivity.—The specificity of the modified assay was confirmed with spring viremia of carp virus, infectious salmon anemia virus, channel catfish virus, koi herpes virus, golden shiner virus, two fathead minnow picornaviruses, and an unknown paramyxo-like virus of white suckers *Catostomus commersonii*. For sensitivity determination, serial dilutions of VHSV-positive cell culture supernatant were tested in duplicate by virus isolation and rRT-PCR on four separate days. The lowest dilution that yielded a positive result was determined to be the limit of detection. The threshold cycle (Ct) for the rRT-PCR was considered as the cycle in which the amplification curve crossed the automatic threshold (Life Technologies) during the exponential growth phase of the curve. The rRT-PCR reaction had a cut-off at 40 cycles, but all samples with Ct values 37–40 were automatically retested. If they remained below 40 cycles, they were called positive.

RESULTS

VHSV Survey

Of 434 contacted, 30 aquaculture producers and wild baitfish harvesters participated in the VHSV survey. The industry impact was probably much higher given that once a water body was certified VHSV-free, anyone with the appropriate license could harvest bait. From April 2010 to June 2011, 115 water bodies were tested for VHSV as part of this survey (Figure 1). The sampling locations with high aquaculture and wild baitfish harvest were well distributed across Minnesota. A total of 7,098 individual fish were collected, comprising 1,420 pooled samples. Approximately 56% ($n = 65$) of the surveyed sites consisted of sport fish, while 44% ($n = 50$) consisted of baitfish. Species collected included walleye ($n = 3{,}375$), spottail shiner ($n = 3{,}000$), black crappie *Pomoxis nigromaculatus* ($n = 285$), muskellunge ($n = 265$), northern pike *Esox lucius* ($n = 80$), bluegill *Lepomis macrochirus* ($n = 60$), yellow perch *Perca flavescens* ($n = 18$),

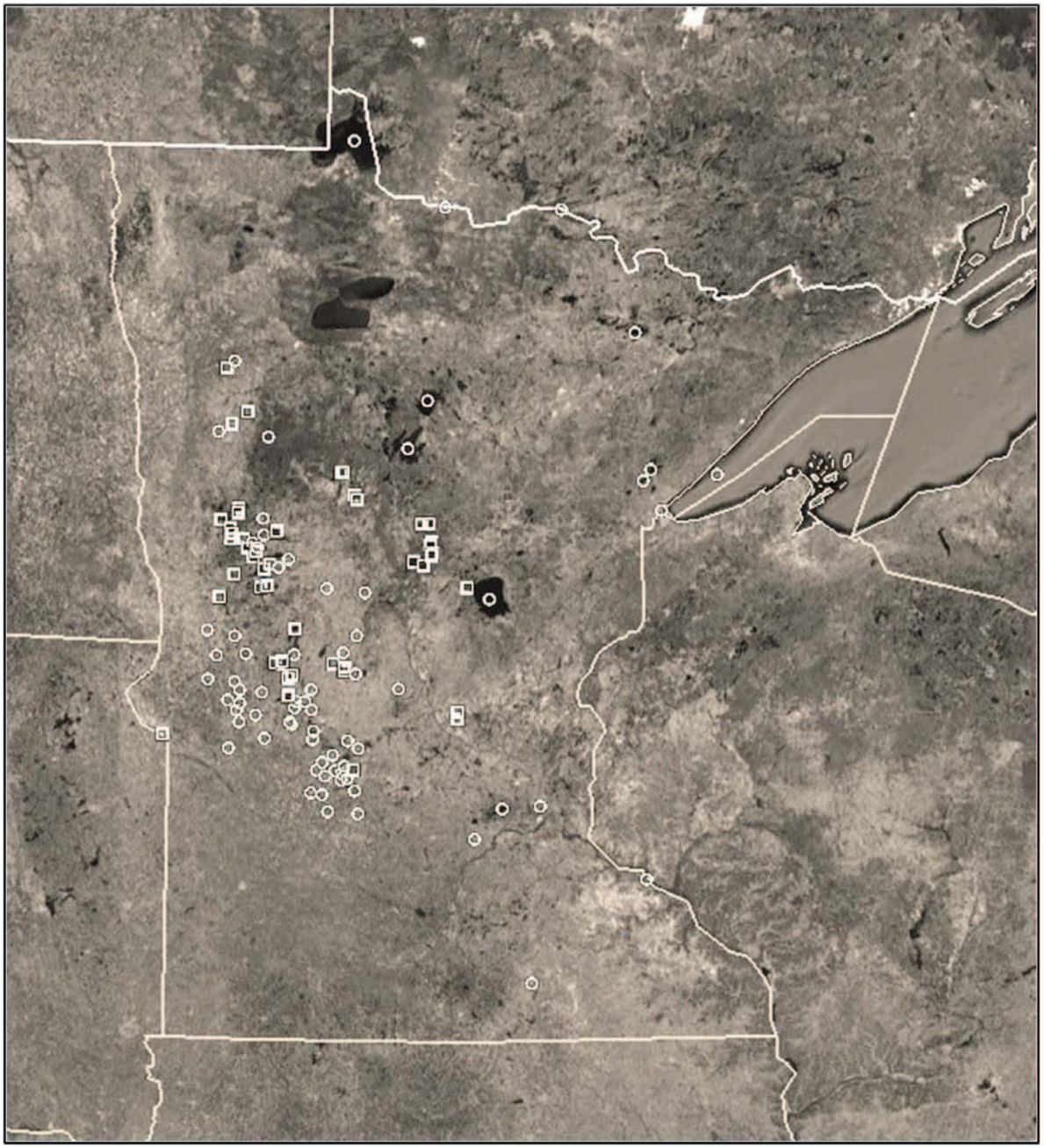

FIGURE 1. Locations of 115 VHSV survey sites in Minnesota collected from April 2010 to June 2011. Circles indicate sport fish ($n = 65$) and squares indicate baitfish ($n = 50$). All locations tested negative for VHSV by virus isolation and rRT-PCR. Image generated with Google Earth. [Figure available online in color.]

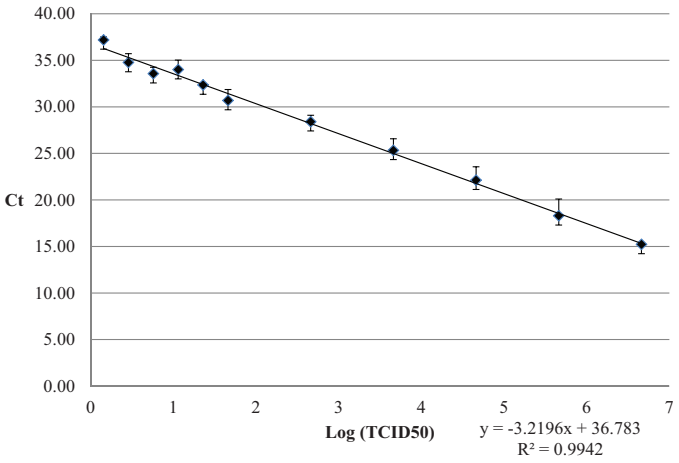

FIGURE 2. rRT-PCR standard curve of dilutions of VHSV, with initial concentration of 4.6×10^6 TCID50 (tissue culture infectious dose with a 50% endpoint). The minimum detection limit was 1.44 TCID50 and had a corresponding Ct of 37.2. The PCR efficiency was 104.8%. Ct = threshold cycle. [Figure available online in color.]

and smallmouth bass *Micropterus dolomieu* ($n = 15$). All sample pools were found negative for VHSV by both virus isolation and rRT-PCR. Throughout the survey, the virus isolation and rRT-PCR assays were 100% accurate in identifying the positive and negative controls. The turnaround time for virus isolation and rRT-PCR was 28–30 d and 2–4 d, respectively.

VHSV rRT-PCR

Analytical specificity.—The rRT-PCR was specific to VHSV and did not cross react with other viruses tested. Although the VHSV-IVb isolate was the only VHSV tested in this validation, sequence alignment and previous data show the primers used can detect all known VHSV strains (Garver et al. 2011).

Analytical sensitivity.—The analytical sensitivity of this assay was comparable with other rRT-PCR assays for VHSV. The standard curve was linear over six logs of virus dilution and had a starting concentration of 4.6×10^6 TCID50 (tissue culture infectious dose with a 50% endpoint) (Figure 2). The correlation coefficient (r) and PCR efficiency were 0.9942 and 104.5%, respectively. The minimum detection limit of the rRT-PCR assay was determined to be a 1.44 TCID50 with a corresponding Ct of 37.2. Compared with this rRT-PCR assay, virus isolation was half as sensitive and had a detection limit of 2.88 TCID50 and a corresponding Ct of 34.8.

DISCUSSION

A newly modified one-step rRT-PCR for the detection of VHSV was used in this study. The assay was shown to be twice as sensitive as the gold standard, virus isolation. Since VHSV can persist in wild fish populations at low levels without producing clinical signs of disease, this improved sensitivity is critically important to prevent the spread of VHSV. In addition, rRT-PCR can significantly decrease the turnaround time and lower labor and laboratory costs. The analytical data generated from this study showed this assay to be an excellent candidate for further evaluation and fit for the purpose of surveillance testing as demonstrated in a 2-year survey of Minnesota waters for VHSV. With nearly 1,500 sample pools tested, no false-positive results were obtained, while both tests accurately identified the negative and positive controls. The results from this survey have certified 115 locations to be VHSV negative, allowing in-state fish movement (for 1 year post inspection).

Aquaculture industry support for this study was very strong in Minnesota, owing to a desire to move fish within days of sample submission rather than having to wait a month. This is essential for any industry that survives on quick turnaround and accurate test results. The survey data have informed management agencies and researchers of the distribution of VHSV in Minnesota. Applications for these data are wide-reaching and will improve selection criteria for future testing, support risk-assessment studies, and ease public concern for this emerging fish disease.

Despite the aforementioned advantages, there are certain limitations of rRT-PCR. One significant limitation is the appropriate interpretation of results. If rRT-PCR is used for surveillance testing, positive rRT-PCR results should remain suspect until confirmed by virus isolation. If virus isolation does not support the suspect-positive result, it should be considered "a population of interest, in need of further testing" or "negative" as it would have been without the use of rRT-PCR. A suspect-positive by rRT-PCR should not result in immediate depopulation or other regulatory action. In the event of conflicting results, other factors should be considered to warrant additional action, such as clinical signs of disease, previous testing history, disease risk factors, or the number of suspect-positive results. Additional action may include, retesting the original material, resampling the population, sequencing the rRT-PCR product, and other confirmatory tests.

One other limitation is proper validation and quality control in the laboratory. Validation standards are outlined by the OIE (2009) and Purcell et al. (2011). While the degree to which an assay is validated depends on its proposed use, the assays used for widespread surveillance testing should be thoroughly scrutinized. The assay used in this study, for example, should undergo an interlaboratory validation to evaluate various platforms, technicians, and diagnostic sensitivity and specificity. Furthermore, proper controls should be used throughout the rRT-PCR assay, including positive and negative controls for RNA extraction and rRT-PCR. These controls were used in this study and increased confidence in the interpretation of results. Endogenous controls were not used in this study, but are recommended to confirm the quality and quantity of nucleic acid (Bland et al. 2012). Other concerns such as laboratory conditions, experience, and capacity are rapidly improving in the United States as technology and demand increases.

Given the threat of aquatic animal pathogens, such as VHSV, new and improved diagnostic tests must be developed, validated, and recognized by agencies for use in surveillance testing. Rapid

detection and quick turnaround time are paramount to identify and effectively control an outbreak. This need has been well demonstrated with the emergence of VHSV-IVb in the Great Lakes. As technology continues to improve and appropriate evaluations are performed, rRT-PCR will become the assay of choice for the detection of viral hemorrhagic septicemia and other emerging diseases of aquatic animals.

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