



Detection and surveillance of viral hemorrhagic septicemia virus using real-time RT-PCR.

II. Diagnostic evaluation of two protocols

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ABSTRACT: Two real-time reverse transcription polymerase chain reaction (rRT-PCR) assays under consideration for deployment to multiple testing laboratories across the USA were evaluated for diagnostic sensitivity and specificity on tissue homogenates obtained from natural and experimental viral hemorrhagic septicemia (VHS)-infected fish. Estimates for diagnostic specificity using virus isolation as the reference method were similar between laboratories regardless of the assay. Diagnostic sensitivity estimates of 0.96 (95% CI: 0.95, 0.97) for Jonstrup et al. (2013)'s assay (*J Fish Dis* 36:9–23) exceeded the diagnostic sensitivity of 0.85 (95% CI: 0.83, 0.87) for Phelps et al. (2012)'s assay (*J Aquat Anim Health* 24:238–243). The Jonstrup rRT-PCR assay is robust as demonstrated by high sensitivity and specificity estimates across laboratories and can be used as a valuable tool for targeted surveillance and for testing of suspect VHSV samples.

KEY WORDS: VHSV · Surveillance · Real-time RT-PCR · Diagnostic sensitivity · Diagnostic specificity

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INTRODUCTION

Rapid and precise detection of viral hemorrhagic septicemia virus (VHSV) is important for disease prevention and containment by reducing the risk

of spread or movement from an infected zone or compartment. Currently, the internationally accepted method for diagnostic, surveillance, and confirmation testing is virus isolation from fish tissue specimens performed on bluegill fry (BF-2), epithe-

lioma papulosum cyprini (EPC), and fathead minnow (FHM) cell cultures with follow-up antibody or nucleic acid based virus identification (OIE 2009). Multiple real-time reverse transcription polymerase chain reaction (rRT-PCR) assays have been developed in order to reduce the amount of time required to obtain a test result. The use of rRT-PCR as a robust targeted surveillance and diagnostic tool is becoming more widely accepted in both aquatic and terrestrial animal health management.

In the USA, VHSV genotype IVa is documented in fish collected from waters in the Pacific Northwest (Hershberger et al. 2010), while VHSV genotype IVb is documented in fish collected from the waters of the Laurentian Great Lakes (Faisal et al. 2012). Many other regions or watersheds in the USA are free from VHSV. The emergence of VHSV IVb in the Laurentian Great Lakes of North America triggered intense surveillance efforts and disease investigations, and VHSV is now considered enzootic in these wild fish populations. Similarly, VHSV is often considered enzootic in the Pacific Northwest, even though the actual disease incidences are unknown. VHSV represents a serious risk to farmed or managed fish populations within the Great Lakes watershed and the Pacific Northwest. In addition, dissemination risks are a consideration when moving fish from these areas to other regions within or outside of the USA (Gustafson et al. 2010, VHSV Expert Panel and Working Group 2010). A rapid and high-throughput diagnostic assay would be extremely useful for controlling VHSV and for facilitating fish trade. However, as testing for VHSV occurs in state, federal, and private laboratories located across the USA and this information is used for disease management, risk analysis, and regulatory purposes, it is imperative to adopt a standardized testing protocol.

In this manuscript, we report on a collaborative side-by-side evaluation of the TaqMan[®] based assay developed by Jonstrup et al. (2013; hereafter 'Jonstrup assay') and the 1-step format of Phelps et al. (2012; 'Phelps assay') that utilizes primers and probe developed by Garver et al. (2011) in an effort to estimate the diagnostic sensitivity and specificity of each test procedure. The 2 assays were selected based on a comparison of analytical performance of 4 assays (Warg et al. 2014, this volume). Eight laboratories evaluated a tissue panel that contained 200 VHSV virus isolation positive and 200 VHSV virus isolation negative tissue homogenate supernatants using standardized rRT-PCR testing protocols.

MATERIALS AND METHODS

Fish samples

VHSV IVa experimentally infected fish

VHSV IVa-injected specific pathogen free Pacific herring *Clupea pallasii* were provided by Dr. Paul Hershberger. Infected fish were euthanized at 12 d post exposure, and the kidney and spleen were removed for a different study (Hershberger et al. 2010). The incision on the fish was closed and the fish was placed in a bag. The fish were stored frozen at -80°C . Frozen fish for processing into whole body homogenates were shipped on dry ice to the National Veterinary Services Laboratories (NVSL).

VHSV IVb wild fish samples

Fish collected as part of a Great Lakes 2010 surveillance effort (E. R. Cornwell et al. unpubl.) were utilized for this study. Tissues (anterior kidney, posterior kidney, spleen, heart, and liver) in 2 ml homogenizing tubes were shipped on frozen gel packs or dry ice to the NVSL.

VHSV IVb experimentally infected fish

VHSV IVb-injected and exposed fish were provided by Dr. A. E. Goodwin. Briefly, largemouth bass *Micropterus salmoides*, bluegill *Lepomis macrochirus*, channel catfish *Ictalurus punctatus*, and grass carp *Ctenopharyngodon idella* were sorted into tanks by species (bluegill were further sorted by size into 2 tanks). Inoculated fish received an intraperitoneal injection of VHSV IVb (1×10^6 TCID₅₀ per ml) and were maintained at 12°C with non-injected fish. All fish in a treatment group were euthanized with MS222 when the first non-injected cohabitant died (an indication of horizontal spread to tank cohabitants). Spleen, kidney, and brain samples were collected, placed in Whirl-Pak[®] bags and immediately frozen at $\leq -80^{\circ}\text{C}$. Tissues were shipped on dry ice to the NVSL.

VHSV negative fish

VHSV negative fish included fish collected from natural water bodies, experimental studies, and diagnostic case submissions. Fish from natural water

bodies outside of the Great Lakes were collected during regional VHSV surveillance efforts. Some of the negative fish samples contained other known pathogens, specifically fathead minnow nidovirus and koi herpesvirus (Table 1).

Tissue panel preparation

All samples were initially tested for VHSV by virus isolation in cell culture at the NVSL prior to inclusion in the test panel (Table 1). Tissue homogenate supernatants were prepared according to the OIE manual (OIE 2009). Briefly, fish tissues were homogenized in Dulbecco's modified Eagle's cell culture medium

supplemented with 2.5% fetal bovine serum, antibiotics, 4.0 mM L-glutamine, 0.006 M sodium bicarbonate, 2 mM sodium pyruvate, and 0.02 M HEPES at a final dilution of 1/10. Homogenates were centrifuged at 2000 to 4000 × *g* (15 min at 4 ± 3°C). Tissue homogenate supernatants were inoculated onto confluent EPC cell cultures and observed regularly for virus-induced cytopathic effects (CPE). When CPE were observed, virus identification by PCR amplification (Hedrick et al. 2003) and direct sequencing of the amplicon were performed. Positive samples selected were observed to induce CPE, and sequencing confirmed the presence of VHSV. Inoculated cultures lacking CPE were sub-cultured 7 to 14 d after inoculation. Subcultures were observed for 14 to 21 d.

Samples were considered negative when no CPE were observed in the secondary cultures. Tissue homogenates were stored in an ultralow freezer (≤−65°C) until aliquoted into panels.

Ten sets containing the numbers 1 to 400 were scrambled using the RAND function and sorting in Microsoft® EXCEL in order to generate tissue sets that lacked any pattern. Each sample in the test panel was assigned a set number and sample number for comparison to the expected result key. Some tissue homogenates were pooled by species in order to have sufficient sample for testing by all laboratories. The negative tissue homogenates were thawed first and centrifuged, and 0.125 ml of the supernatant aliquoted into appropriately labeled tubes and immediately refrozen in boxed sets. Positive tissue homogenates were thawed second and aliquoted in the same manner. Panels were shipped to participating laboratories on dry ice. Virus isolation was repeated on tissue homogenates when thawed for dispensing into aliquots.

RNA extraction and real-time RT-PCR

Each tissue homogenate was extracted 1 time by participating labs using the MagMAX™-96 Viral RNA Isolation Kit (Ambion® kit AM1836,

Table 1. Tissue panel samples. FHMNV: fathead minnow nidovirus; KHV: koi herpesvirus

Sample number(s)	Common name	Single fish or pool	Source
Positive samples			
1–24, 26–36, 40, 45–62	Round goby	Single	Wild Great Lakes
38, 44	Yellow perch	Single	Wild Great Lakes
39, 41, 42	Rock bass	Single	Wild Great Lakes
43	Pumpkinseed	Single	Wild Great Lakes
25	Smallmouth bass	Single	Wild Great Lakes
37	Bluegill	Single	Wild Great Lakes
66, 70, 73	Bluegill	Pool of 2	Exp IVb -contact
63–65, 67–69, 71, 72, 74–101	Bluegill	Single	Exp IVb -contact
102–110	Largemouth bass	Single	Exp IVb -contact
111–119	Channel catfish	Single	Exp IVb -contact
120–123, 136–139	Muskellunge	Single	Injected exp IVb
124–130	Muskellunge	Pool of 2	Injected exp IVb
131–132	Muskellunge	Pool of 3	Injected exp IVb
133	Muskellunge	Pool of 4	Injected exp IVb
134–135	Muskellunge	Pool of 5	Injected exp IVb
140–200	Pacific herring	Single	Injected exp IVa
Negative samples			
201	Largemouth bass	Pool of 5	Exp neg control
202	Bluegill	Pool of 5	Exp neg control
203	Bluegill	Pool of 5	Exp neg control
204	Channel catfish	Pool of 5	Exp neg control
205	Channel catfish	Pool of 3	Exp neg control
206–207	Grass carp	Single	Exp neg control
208–257	Pacific herring	Single	Exp neg control
258–280	Fathead minnow	Single	Injected exp FHMNV
281	Koi	Single	KHV positive
282–381	Walleye	Pool of 5	Wild fish negative
382–383	Smallmouth bass	Pool of 5	Wild fish negative
384	Rock bass	Pool of 5	Wild fish negative
385–393	Yellow perch	Pool of 5	Wild fish negative
394	Hybrid bluegill	Pool of 5	Wild fish negative
395–396	Bluegill	Pool of 5	Wild fish negative
397–398	Largemouth bass	Pool of 5	Wild fish negative
399–400	Fathead minnow	Pool of 5	Wild fish negative

Life Technologies) using either an automated or manual system. The automated and manual extraction processes provide consistent nucleic acid yields (Shah et al. 2009). The same RNA extracted from each tissue homogenate sample was evaluated by both rRT-PCR assays. Both assays use a 1-step approach with different pan-VHSV primers and probes that target the nucleoprotein. Laboratories followed standardized testing protocols supplied to all participants. Detailed information on each assay can be found in Jonstrup et al. (2013) and Phelps et al. (2012). Briefly, for the Jonstrup assay a 25 μ l reaction mixture contained the Quantitect RT-PCR Buffer and RT-PCR Enzyme (Qiagen), FAM-labeled TaqMan probe (5'-6-FAM-TAG AGG GCC TTG GTG ATC TTC TG-BHQ1), primers (5'-AAA CTC GCA GGA TGT GTG CGT CC-3' and 5'-TCT GCG ATC TCA GTC AGG ATG AA-3'), and the RNA sample. The thermal cycling protocol consisted of 30 min at 50°C, 15 min at 95°C, followed by 40 cycles of 15 s at 94°C, 40 s at 60°C, and 20 s at 72°C. For the Phelps assay, a 25 μ l reaction mixture contained Path-ID Multiplex RT-PCR buffer and Path-ID Multiplex enzyme mix (Life Technologies), FAM-labeled MGB Probe (5'-Fam-TAC GCC ATC ATG ATG AGT-3'), primers (5'-ATG AGG CAG GTG TCG GAG G-3' and 5'-TGT AGT AGG ACT CTC CCA GCA TCC-3'), and the RNA sample. The thermal cycling protocol consisted of 10 min at 45°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

Laboratories ('Labs') participating in this comparison had different levels of high-throughput or rRT-PCR testing experience (Table 2 in Warg et al. 2014) ranging from extensive experience (high) to limited experience (recently trained). In Labs A, D, and E, personnel were experienced with high-throughput testing and with conducting rRT-PCR. In Labs B and H, personnel were experienced with large numbers of samples and with conventional RT-PCR, but were recently trained to perform real-time assays. Labs C and G have dual roles as both diagnostic and research laboratories with some experience with both high-throughput testing and rRT-PCR. Lab F also has dual function being involved in both research and diagnostics; in this laboratory, the technicians were recently trained in high-throughput testing and to perform real-time assays. Specific real-time PCR machines and software used in this study (Table 2 in Warg et al. 2014) included the ABI 7500 software version 1.4 or software version 2.0 (ABI, Life Technologies), BioRad iCycler iQ™ software version 3.1 (BioRad), and Mastercycler® EP Realplex software version 1.5 or software version 2.2 (Eppendorf).

Data analysis parameters were standardized (Warg et al. 2014). Briefly, the auto Ct algorithm was utilized to set the baseline and threshold parameters for ABI platforms and software. If the baseline was set too low (higher background on a run), a second analysis was performed using auto threshold and manual baseline settings (3 to 15 cycles). The Auto Ct algorithm was used for the BioRad iCycler iQ™ software version 3.1. For the Mastercycler® EP Realplex software version 1.5, the best-fit algorithm was used to set the baseline and threshold. Thresholds were manually established for the Mastercycler® EP Realplex software version 2.2 based on a set percentage of the plateau of the low positive amplification control.

Diagnostic sensitivity, specificity, and reproducibility

Diagnostic sensitivity (DSe) and specificity (DSp) estimates using virus isolation as the 'true' infection status were estimated. A sample was classified as PCR positive if a cycle threshold (Ct) value was produced. A generalized linear model with a logit link (McCullagh & Nelder 1989) was fit to the number of correct test results (across laboratories) for each sample. An estimate of the mean, μ , was used to determine the sensitivity (or specificity) as $1 / [1 + \exp(-\mu)]$. Confidence intervals were obtained using the delta method. Qualitative test results (positive or negative) for each sample were aligned to gauge the agreement of the test result for the same assay on the same sample in different laboratories. Ct values from positive amplification controls were used to monitor assay performance.

RESULTS

Diagnostic sensitivity

The percentages of correct positive classifications by laboratory for the portion of positive tissue homogenates that tested positive were calculated (Table 2). Out of the 200 samples positive by virus isolation for VHSV, 185 to 199 tested positive by the Jonstrup assay and 128 to 196 tested positive by the Phelps assay. Sensitivity was estimated to be 0.96 (95% CI: 0.95, 0.97) for the Jonstrup assay and 0.85 (95% CI: 0.83, 0.87) for the Phelps assay. Test results were aligned (Fig. 1) to evaluate whether any particular samples produced false negative test

Table 2. Percentage of correct classifications by laboratory. VI: virus isolation reference standard; DSe (%): diagnostic sensitivity; DS_p (%): diagnostic specificity

Method Lab	VI + / PCR +	VI + / PCR -	VI - / PCR +	VI - / PCR -	DSe (%)	DS _p (%)
Jonstrup et al. (2013)						
A	196	5	0	200	97.5	100.0
B	198	2	0	200	99.0	100.0
C	191	9	8	192	95.5	96.0
D	196	4	0	200	98.0	100.0
E	199	1	0	200	99.5	100.0
F	190	10	26	174	95.0	87.0
G	188	12	3	197	94.0	98.5
H	185	15	12	188	92.5	94.0
Phelps et al. (2012)						
A	173	27	0	200	86.5	100.0
B	196	4	0	200	98.0	100.0
C	128	71	2	197	64.3	99.0
D	188	12	0	198	94.0	100.0
E	165	35	0	200	82.5	100.0
F	178	22	0	200	89.0	100.0
G	144	56	0	200	72.0	100.0
H	188	12	16	184	94.0	92.0

results in multiple laboratories. Three samples (3, 108, and 116) were reported as false negatives when evaluated by the Jonstrup assay by most laboratories. Other samples with false negative results reported by 2 or more laboratories included sample 77 (4 laboratories), samples 70 and 81 (3 laboratories), and samples 65, 71, 94, 138 and 160 (2 laboratories). False negative results were reported for both VHSV IVa (5 samples) and VHSV IVb (24 samples). The number of injected VHSV IVb (2 samples) false negative samples was similar to the number of injected IVa (5 samples) false negative samples. The remaining VHSV IVb false negative samples included 7 natural infection and 15 experimental infection contact animals. An additional 18 samples were reported as false negative by a single laboratory. The number of virus isolation positive samples with false negative reports when evaluated by the Phelps assay was high (79 samples had at least 1 laboratory reporting them as false negative) as compared to the Jonstrup assay (29 samples). Samples 3, 108, and 116 were also reported as false negatives by the Phelps assay.

Diagnostic specificity

The percentages of negative classifications by laboratory for the portion of negative tissue homogenates that tested negative were calculated

(Table 2). Out of the 200 samples negative by virus isolation for VHSV, 174 to 200 tested negative by the Jonstrup assay and 184 to 200 by the Phelps assay. Specificity was estimated to be 0.97 (95% CI: 0.97, 0.98) for the Jonstrup assay and 0.99 (95% CI: 0.98, 0.99) for the Phelps assay. Four laboratories (A, B, D, and E) reported no false positive test results for either assay. False positive results included both low and high Ct values. No single sample was reported as false positive by multiple laboratories. Two labs (F and H) reported the majority of false positives for the Jonstrup assay; a single lab (H) had the majority of false positives for the Phelps assay. Lab H also experienced a high number of false negatives with the Jonstrup assay.

DISCUSSION

The objective of this study was to evaluate (side by side) the diagnostic performance of 2 assays under consideration for deployment to a network of independent laboratories that provide surveillance, diagnostic, and fish facility monitoring testing for VHSV. The tissue samples utilized in this comparison were selected to closely match the types of samples a diagnostic laboratory might receive for routine testing in terms of species, sample matrix, attribute being measured, and target concentrations. In order to reduce but not eliminate inhomogeneity and instability concerns, tissue homogenate supernatants were selected as the testing matrix. Extensive homogeneity and stability testing was not possible due to sample size limitations. However, in such cases the use of this type of sample is still useful as long as this uncertainty is considered during the evaluation of results (ISO 2010).

Estimates of diagnostic sensitivity and specificity from this study are most likely biased as the true classification for each sample is based on an imperfect test. The trueness of the classification does not preclude comparing assay performance across laboratories on these samples. Further, the reference test is detecting infectious virus, while the PCR assays detect the presence of nucleic acid. The performance (robustness) of the assays was compared across the laboratories using the percentage of samples with

the correct classification. This comparison is also impacted by inherent biases which are identical for all 8 laboratories. Therefore, a comparison of the 2 different rRT-PCR assays performance on the same tissue panel by testing laboratories is not likely to be impacted by these particular inherent biases. In this multi-laboratory evaluation of rRT-PCR assays for VHSV detection, the Jonstrup assay (Jonstrup et al. 2013) outperformed the Phelps assay (Phelps et al. 2012) for diagnostic sensitivity, but assay performances were similar for diagnostic specificity.

The Phelps assay is a modification of the Garver et al. (2011) VHSV rRT-PCR 2-step assay to a 1-step approach (Phelps et al. 2012). Diagnostic sensitivity estimates for the Phelps assay of <90% by 5 out of the 8 participating laboratories was lower than expected based on analytical published data of Phelps et al. (2013) and comparisons of the limit of detection (LOD) on representative isolates from each genotype of VHSV for this assay (Warg et al. 2014). A comparison of analytical sensitivity for the Phelps assay and the Garver assay reported similar LODs (Warg et al. 2014). However, efficiency estimates for the Phelps assay (102–151% on VHSV MI03 RNA standards) were suboptimal (optimal being 90–104%).

Virus isolation is an imperfect standard, and this was taken into consideration when assigning the infection status of a sample and evaluating assay comparison data (Fig. 1). Virus isolation was repeated on all tissue homogenate supernatants when thawed for aliquoting into the test panels. Virus was recovered from 134 of the 200 samples that originally tested positive for VHSV. This is not unexpected as material frozen (whole fish, tissue homogenate, or supernatant) and freeze–thaw events can impact the ability to recover replicating virus from a diagnostic sample (Meyers et al. 1999, Arkush et al. 2006, Hervé-Claude et al. 2008), but have less impact on the ability to detect viral RNA (Phelps et al. 2013). All 200 negative samples tested negative on repeat virus isolation testing. Samples 3, 108, and 116 showed ‘false negative’ results with both PCR assays (Fig. 1) in multiple but not all laboratories. Samples 108 (largemouth bass) and 116 (channel catfish) were both fish cohabited in tanks with injected fish (Table 1) and were negative for virus isolation on repeat testing. Sample 3 was a naturally infected round goby that was positive for virus isolation on repeat testing. False negative PCR results were obtained by the Jonstrup assay for 6 cohabitant bluegill (samples 65, 70, 71, 77, 81, 84) and 2 injected fish (muskellunge 138 and herring 160); these samples were negative for virus isolation on repeat test-

ing. Samples reported as false negative by the Phelps assay included wild, cohabitants, and injected fish. False positive classification is equally a concern. The number of false positives was low by both assays. Sample history and a review of samples testing false positive do not suggest misclassification of infection status.

On the basis of this study, the rRT-PCR assay of Jonstrup et al. (2013) can be used as a valuable tool when surveillance or suspect VHSV samples are submitted to a laboratory for testing. In addition, the high throughput capacity and the speed of the assay will allow rapid identification of VHSV affected farms or populations.

Acknowledgements. We thank Søren Jonstrup, formerly of the Section for Fish Diseases, National Veterinary Institute (Denmark), for sharing his testing protocols prior to publication; Paul Hershberger of the Marrowstone Marine Field Station, United States Geological Survey, for providing VHSV IVa positive tissues; and Mary Lea Killian of the Diagnostic Virology Laboratory, National Veterinary Services Laboratories, and Gael Kurath of the USGS Western Fisheries Research Center, for helpful discussions. This work was funded by USDA. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.

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*Editorial responsibility: Mark Crane,
Geelong, Victoria, Australia*

*Submitted: July 31, 2013; Accepted: April 29, 2014
Proofs received from author(s): July 31, 2014*