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Detection and surveillance of viral hemorrhagic septicemia virus using real-time RT-PCR. I. Initial comparison of four protocols

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ABSTRACT: Eight laboratories worked collectively to evaluate 4 real-time RT-PCR (rRT-PCR) protocols targeting viral hemorrhagic septicemia virus (VHSV) being considered for deployment to a USA laboratory testing network. The protocols utilized previously published primers and probe sets developed for detection and surveillance of VHSV. All participating laboratories received and followed a standard operating protocol for extraction and for each of the rRT-PCR assays. Performance measures specifically evaluated included limit of detection (defined as the smallest amount of analyte in which 95% of the samples are classified as positive), analytical specificity, assay efficiency across genotype representatives, within- and between-plate variation within a laboratory, and variation between laboratories using the same platform, between platforms, and between software versions. This evaluation clearly demonstrated that the TaqMan®-based assay developed by Jonstrup et al. (2013; *J Fish Dis* 36:9–23) produced the most consistent analytical performance characteristics for detecting all genotypes of VHSV across the 8 participating laboratories.

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

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) is the causative agent of a highly contagious disease of both saltwater and freshwater fish in the northern hemisphere. VHSV is a novirhabdovirus (*Rhabdoviridae*) (Tordo et al. 2005) that is listed as a notifiable pathogen by the World Organization for Animal Health (OIE). The severity of infection and its outcome varies by fish species, age, immune status, and prevailing

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environmental conditions. Dependent on the species of fish, infection may result in substantial mortality (Smail & Snow 2011, Kim & Faisal 2010a,b,c, Groocock et al. 2012). VHSV can become endemic in fish populations, with some individual fish serving as carriers of the virus and periodically shedding the virus, which causes repeat episodes of disease in naïve fish populations (Hershberger et al. 2010, Kim & Faisal 2012). Since its emergence in the Laurentian Great Lakes of North America, the novel sublineage of VHSV genotype IV has caused several large-scale die-offs of wild fish and spread into the 5 Great Lakes, a number of inland lakes, and several streams (Elsayed et al. 2006, Gagné et al. 2007, Groocock et al. 2007, Lumsden et al. 2007, Cornwell et al. 2011, 2012). VHSV is now endemic in the Great Lakes and represents a serious risk to farmed fish populations within the Great Lakes watershed as well as a risk for dissemination of the virus to other watersheds within or outside the USA by movement of fish (reviewed by Faisal et al. 2012).

The current gold standard for detection of VHSV in either clinical or non-clinical fish is virus isolation in cell culture followed by virus identification (AFS-FHS 2010, OIE 2012). Virus isolation in cell culture is a long and labor-intensive process. Multiple real-time RT-PCR (rRT-PCR) assays with rapid turnaround time have been developed and suggested for use in VHSV surveillance efforts or for routine diagnosis of viral hemorrhagic septicemia (VHS) (Chico et al. 2006, Liu et al. 2008, Matejusova et al. 2008, 2010, Cutrín et al. 2009, Hope et al. 2010, Garver et al. 2011, Jonstrup et al. 2013, Pierce et al. 2013a,b). However, in order to consider utilization of any VHSV rRT-PCR assay in surveillance or diagnostic activities where testing is conducted in multiple laboratories and where the results from multiple laboratories may be used to support declarations of freedom from the pathogen, the testing protocol must be standardized, robust, and performance estimates well characterized.

The definition of what constitutes a standardized testing protocol varies. For rRT-PCR assays, the OIE Aquatic Diagnostic Manual frequently lists primers, probe, and cycling conditions but lacks many of the details that can influence a test's outcome. Quality controls (such as extraction and amplification controls and acceptable cycle threshold [Ct] value ranges for positive extraction and amplification controls) and interpretation criteria need to be defined in the standardized testing protocol. To this end, the USDA-APHIS coordinated efforts of 8 laboratories to compare the analytical performance of 4 rRT-PCR protocols that utilized 3 previously published primers and probe sets (Hope et al. 2010, Garver et al. 2011, Phelps et al. 2012, Jonstrup et al. 2013) following OIE validation guidelines. In addition, the purpose of this comparison is exclusively for detection (an unequivocal positive or negative test result) of VHSV for diagnosis or surveillance, although the assays could be used for relative quantitation purposes. The participating laboratories received and followed a standard operating protocol for extraction and for each of the rRT-PCR assays. This paper reports the criteria used for the comparison of assay performance across participating laboratories as well as the results. Performance measures specifically investigated included limit of detection (LOD), defined as the smallest amount of analyte in which 95% of the samples are classified as positive, analytical specificity, estimates of assay efficiency across genotype representatives, within- and between-plate variation within a laboratory, variation between laboratories using the same platform, between platforms, and between software versions. In addition, both the LOD and the efficiency estimates can be used as indicators of laboratory/technician sensitivity differences related to sample handling (serial dilutions of virus stocks) within a testing laboratory. Performance measures were used collectively to select assays for further evaluation and to make recommendations on the suitability of each of these assays for surveillance and diagnostic purposes.

MATERIALS AND METHODS

Samples and standards

Virus strains representing the 4 different VHSV genotypes were used in this study (Table 1); these included reference viruses and field isolates. Tissue culture infectious dose 50% ($TCID_{50}$) per ml titers were calculated for each VHSV stock by the Spearman-Kärber method as modified by Finney (1978) in a 96-well plate format using 10-fold serial dilutions. Fish tissue and fish pathogens (see Table 3) were used to evaluate analytical specificity (exclusivity). Positive amplification controls (PAC) included 2 different sets (3 concentrations in each set) of VHSV-relevant RNA standards. A VHSV-infected round goby RNA standard set (Hope et al. 2010) was used during Phase I testing, and a VHSV MI03 isolate RNA standard set was used during Phase II testing. Each laboratory used its own VHSV reference virus as a positive extraction control. Negative extraction controls were diluents (cell culture medium) utilized at each participating laboratory. Negative amplification control was RNA elution buffer or water.

RNA extraction

The MagMAX™-96 Viral RNA Isolation Kit (Ambion® kit 1836, Life Technologies) was used to extract nucleic acids using either an automated or manual system (Shah et al. 2009). Automated systems included MagMAX™ Express (Applied Biosystems (ABI), Life Technologies), or Kingfisher (Thermo Fisher Scientific), or BioSprint 96 (Qiagen) magnetic particle processors; and manual systems used an Ambion magnetic plate stand following the manufacturer's instructions. Protocol AM1836 DW 50 v2 Aqua (ABI, Life Technologies) was followed for the automated extraction.

rRT-PCR

Three previously published VHSV-specific primer and probe sets (identified as the Garver, Hope, and Jonstrup assays in this study) were selected based on published performance characteristics, scientific validity data including performance in species and population to be tested, and international acceptance (Hope et al. 2010, Garver et al. 2011, Jonstrup et al. 2013). The Hope and the Jonstrup assays are single-tube assays where both reverse transcription (RT) and PCR occur in a single tube using primers specific for a fragment of the nucleoprotein (N) gene of VHSV. The Garver assay is a 2-step assay that requires RT of RNA to cDNA using random primers in 1 tube, followed by real-time PCR in a second tube. A fourth assay (identified as the Phelps assay in this study) utilized the Garver primers and probe in a single tube format (Phelps et al. 2012).

Testing phases

The purpose of Phase I was to establish performance characteristics on a single platform and software package to be used as the 'reference standard platform.' Criteria for selection of laboratories for this phase were those conducting VHSV surveillance, movement, or confirmation testing on fish collected from the Great Lakes watershed and had the reference platform and software in the laboratory (Table 2). Three laboratories (A, B, and C) tested 9 VHSV isolates, representing the 4 genotypes, with the 4 different rRT-PCR methods on the ABI 7500 — software version 1.4 platform (ABI, Life Technologies). From a single stock for each isolate, each laboratory created 3 separate 10-fold serial dilutions spanning concentrations 10^{-1} through 10^{-8}. Total RNA

Table 1. Viral hemorrhagic septicemia virus (VHSV) reference isolates used for establishing limit of detection and amplification efficiency

Virus isolate	Genotype	Location	Host	Year
DK5151	Ia	Rindsholm, Denmark	Rainbow trout	1992
1p53	II	Baltic Sea	Herring	1996
F13.02.97	III	Farmed; Ireland	Turbot	1997
Makah	IVa	Hatchery; USA	Coho salmon	1988
ME03	IVa	Atlantic Ocean	Herring	2003
2000-149	IVc	Ruisseau George-Collette, Canada	Mummichog	2000
MI03	IVb	Lake St. Clair, USA	Muskellunge	2003
2004-175	IVc	French River, Canada	Brown trout	2004
FPL2006-005	IVb	St. Lawrence River, USA	Round goby	2006

Year was corrected after publication

Table 2. Laboratory information. PCR experience levels were categorized as High: extensive experience with high-throughput rRT-PCR testing for multiple pathogens; Some: recent experience with high-throughput VHSV rRT-PCR testing; Recently trained: experience with high sample testing numbers but recently trained to perform rRT-PCR

Lab	Platform	Manufacturer	Software version	Function	rRT-PCR experience
A	ABI 7500	ABI, Life Technologies	1.4	Diagnostic	High
B	ABI 7500	ABI, Life Technologies	1.4	Diagnostic	Recently trained
C	ABI 7500	ABI, Life Technologies	1.4	Research and diagnostic	Some
D	ABI 7500	ABI, Life Technologies	1.4	Diagnostic	High
E	Mastercycle® EP Realplex	Eppendorf	1.5	Diagnostic	High
F	Mastercycle® EP Realplex	Eppendorf	2.2	Research and diagnostic	Recently trained
G	BioRad iCycler iQ™	BioRad	3.1	Research and diagnostic	Recently trained
H	ABI 7500	ABI, Life Technologies	2.0	Diagnostic	Recently trained

was extracted from 50 µl of each replicate 10-fold serial dilution. Individual RNA samples were analyzed once by each assay, and Ct values were recorded for use in determining test performance characteristics. Controls for every run included negative extraction controls, negative amplification controls, positive extraction controls, and positive amplification controls. Estimates for LOD were considered in conjunction with the estimates for efficiency when comparing assays.

The purpose of Phase II was to establish performance characteristics in laboratories (hereafter 'Labs') that utilized alternative platforms and software. Criteria for selection of labs for this phase were those conducting VHSV surveillance or movement testing on fish collected from outside the Great Lakes watershed and/or had a different platform and software in the lab. Five labs (D, E, F, G, and H) tested 4 VHSV genotype IV isolates using 2 different rRT-PCR methods (Jonstrup and Phelps methods) as outlined in Phase I. Platforms and software used for this phase are listed in Table 2. Phase II data allowed for a comparison of platforms or an individual lab's performance with the reference standard platform. Comparison of Labs A, B, C, and D was a direct comparison of labs or individuals performing the assay in a lab as the platform is the same in each of these labs and a single individual performed the test in each lab (lab and technical operator are confounded). The ABI 7500 (software version 1.4) platform used by Labs A, B, and C in Phase I testing was considered the 'reference standard platform'; Lab D also used the reference standard platform during Phase II testing. Phase II results from Labs D, E, F, G, and H (other platforms or individuals) were compared to Phase I results from Labs A, B, and C. Lab F had 2 individuals (F_1 and F_2) perform the testing.

Determination of assay performance characteristics

Data interpretation

The Ct value of a rRT-PCR reaction is defined by the threshold line set within the exponential phase of the logarithmic scale amplification plot, and the baseline reflects the background or noise in the reactions. For the reference standard platform (ABI 7500, software version 1.4), the auto Ct algorithm, which calculates baseline and threshold parameters based on the assumption that data sets will exhibit characteristic amplification curves, was utilized. After each analysis, the baseline and threshold parameters were reviewed. When 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. Threshold setting for the Mastercycler® EP Realplex software version 2.2 was problematic, as neither the best fit nor noiseband algorithm (software defined) produced repeatable results. Thresholds were manually established for the Mastercycle® EP Realplex software version 2.2 based on a set percentage of the plateau of the low positive amplification control (ABI recommendation to optimize consistency across platforms and for correct data interpretation for low copy number samples).

Analytical sensitivity

The LOD was determined as the concentration ($TCID_{50}$ per ml titer) corresponding to the last serial dilution in which all 3 replicates tested positive. A regression analysis was conducted (Ct versus $-\log_{10}$ dilution) on the data from each laboratory for each isolate by rRT-PCR method using only the data within the LOD. The slope of the regression line was used to estimate the amplification efficiency (AE) and was reported as a percentage: $AE = 100 \times (10^{1/slope} - 1)$. Comparison of each laboratory's performance characteristics (LOD and AE) by isolate and method were performed.

Repeatability and reproducibility

Linear mixed effects models were fit to Ct values obtained for the VHSV MI03 RNA standard set (provided to laboratories) produced by each assay for each laboratory. The models included a fixed dilution effect, a random plate effect, a random dilution by plate effect, and a random error. The square root of the variance of the random error was reported as the within-plate variability. The square root of the sum of the variances of the random plate and random dilution by plate effects was reported as the between-plate variability. The total variability was reported as the square root of the sum of all variance components (Vardeman & Jobe 1999).

Analytical specificity

Labs D, E, F, and G conducted specificity testing. Reference cultures or field isolates (Table 3) were diluted to obtain a high concentration (approximately 10 000 genome copies) and a low concentration (approximately 100 genome copies) for nucleic acid extraction. Each nucleic acid sample was tested in duplicate by both the Jonstrup and the Phelps assays.

Table 3. Diagnostic matrices used to demonstrate analytical specificity

Strain or species	Common name	Testing lab(s)
Virus		
Aquareovirus A	Chum salmon reovirus	G
Aquareovirus B	Green river chinook reovirus	G
Aquareovirus B	Eel lake reovirus	G
Aquareovirus C	Golden shiner virus	G
Aquareovirus D	American grass carp reovirus	G
Cyprinid herpesvirus 3	Koi herpesvirus	E
Fathead minnow nidovirus		G
Ictalurid herpesvirus 1	Channel catfish virus	G
Infectious hematopoietic necrosis virus		D, E
Infectious pancreatic necrosis virus		D, E, F
Largemouth bass virus		D, E, F
Picornavirus		G
Spring viremia carp virus		D
Bacteria		
Aeromonas hydrophila		E, F
Aeromonas salmonicida		D, F
Flavobacterium psychrophilum		D, F
Flavobacterium columnare		F
Pseudomonas aeruginosa		D, E
Reinbacterium salmoninarum		F
Shewanella putrefaciens		E, F
Streptococcus uberis		E
Yersinia ruckeri		D, F
Fish		
Ameiurus nebulosus	Bullhead	D
Catostomus commersonii	White sucker	D
Cyprinus carpio	Common carp	D
Esox masquinongy × *Esox lucius*	Tiger muskellunge hybrid	D
Etheostoma exile	Iowa darter	D
Hybognathus hankinsoni	Brassy minnow	D
Lepomis cyanellus	Green sunfish	D
Lepomis macrochirus	Bluegill	D
Micropterus dolomieu	Smallmouth bass	D
Morone chrysops	White bass	D
Perca flavescens	Yellow perch	D
Pimephales promelas	Fathead minnow	D
Pomoxis nigromaculatus	Black crappie	D
Sander vitreus	Walleye	D

RESULTS

Phase I

LOD estimates (Table 4) on isolates representative of VHSV genotypes varied by isolate and assay as expected given the nucleic acid variation between genotypes and different starting concentrations of the isolates. There was not a single laboratory that had the best sensitivity (LOD) across all isolates for an assay or across assays. The Hope assay did not detect the genotype I virus (DK5151). Both the Garver and the Hope assays only detected a high concentration (4–5 logs of virus) of genotype II virus (1p53). Regression analysis (Table 5) of the data for 3 labs (A, B, and C) revealed similar AE for an assay regardless of an isolate's genotype within a lab when there was a positive detection. Linear regression analysis on the data set for several isolates tested with the Phelps assay in Lab C did not hold a constant linear relationship between Ct value and dilution across all concentrations tested (Fig. 1). The Jonstrup assay consistently detected low concentrations of all genotypes and had high efficiency estimates (87–97; 92–98; 97–109) in all 3 labs. Plots of the regression analysis (data not shown) as it relates to the cut-off values utilized in all assays suggest that cutoff values exceeded the linear relationship between the Ct value and $-\log_{10}$ dilution of the virus stock. The Jonstrup and the Phelps assays were chosen for Phase II testing, as both assays detected all genotypes of VHSV known to date. The Phelps assay was chosen over the Garver assay, as 1-step assays are technically more convenient to perform and have higher throughput capacity and did a better job at detecting genotype representatives used in this study.

Phase II

Analytical sensitivity

Five labs (D, E, F, G, and H) determined LOD for genotype IV isolates only. A regression analysis to obtain amplifica-

Table 4. Limit of detection estimates are defined as the concentration corresponding to the last serial dilution in which all 3 replicates tested positive. Titer is tissue culture infective dose ($TCID_{50}$) ml^{-1}; ND: not detected; (–) virus not tested

Method	Isolate ID (genotype)								
Lab	DK5151 (Ia)	1p53 (II)	F13.02.97 (III)	Makah (IVa)	ME03 (IVa)	2000-149 (IVc)	MI03 (IVb)	2004-175 (IVb)	FPL2006-005 (IVb)
Titer	8.4	7.1	8.2	7.1	7.3	8.3	8.1	6.1	8.4
Hope et al. (2010)									
A	ND	5.1	2.2	2.1	1.3	3.3	3.1	1.1	3.4
B	ND	5.1	2.2	2.1	1.3	2.3	2.1	0.1	1.4
C	ND	4.1	2.2	2.1	1.3	2.3	2.1	0.1	1.4
Garver et al. (2011)									
A	3.4	6.1	3.2	3.1	2.3	3.3	3.1	1.1	2.4
B	2.4	ND	4.2	3.1	2.3	3.3	2.1	1.1	3.4
C	3.4	6.1	4.2	2.1	2.3	2.3	2.1	1.1	2.4
Phelps et al. (2012)									
A	2.4	3.1	3.2	2.1	1.3	2.3	2.1	0.1	2.4
B	2.4	3.1	3.2	2.1	1.3	2.3	1.1	1.1	2.4
C	2.4	3.1	3.2	3.1	2.3	3.3	3.1	2.1	2.4
D	–	–	–	2.1	1.3	2.3	–	–	2.4
E	–	–	–	3.1	2.3	3.3	–	–	3.4
F	–	–	–	4.1	3.3	4.3	–	–	4.4
G	–	–	–	4.1	3.3	4.3	–	–	3.4
H	–	–	–	2.1	1.3	3.3	–	–	2.4
Jonstrup et al. (2013)									
A	2.4	1.1	2.2	2.1	2.3	2.3	2.1	1.1	3.4
B	2.4	1.1	2.2	2.1	2.3	3.3	2.1	1.1	3.4
C	1.4	1.1	1.2	3.1	1.3	2.3	2.1	0.1	2.4
D	–	–	–	2.1	1.3	2.3	–	–	2.4
E	–	–	–	2.1	1.3	2.3	–	–	2.4
F	–	–	–	4.1	3.3	3.3	–	–	3.4
G	–	–	–	3.1	1.3	2.3	–	–	2.4
H	–	–	–	3.1	1.3	3.3	–	–	2.4

tion efficiency estimates was conducted on the data from each laboratory for each assay–isolate combination (Table 5). Efficiency estimates based on regression analysis were used to evaluate the performance of each assay in participating laboratories since Ct values cannot be used when comparing experiments where different platforms and software are used. In these instances, the AE is based on a model with only those concentrations in which it was reasonable to assume a constant linear relationship.

Lab D used the same platform and software as Labs A, B, and C, but did not meet both of the selection criteria for Phase I. Lab H used the same platform as the reference standard platform with the exception of a newer version of the software. Lab E used a different platform/software from the reference standard platform. The LOD estimates (Fig. 2) for both the Jonstrup and the Phelps assays were similar across Labs A to E and H. For the Jonstrup assay, Labs D and E consistently had the best LOD (lowest titer of virus detected).

Lab F had an LOD estimate (Fig. 2) 1 log higher than the largest observed estimate across Labs A to D for 2 of the isolates and was similar to the worst observed estimate across Labs A to D for the other 2 isolates for the Jonstrup assay. The LOD estimates for Lab F across all isolates were 1 log worse than the observed estimates across Labs A to D for the Phelps assay.

Lab G used a different platform/software than any of the other participating labs. For the Jonstrup assay, the LOD estimates (Fig. 2) were similar to observed estimates across labs A to E and H. For 3 of the 4 isolates, LOD estimates were 1 log worse than the largest LOD estimate provided by Labs A, B, C, or D using the Phelps assay.

Efficiency estimates (Table 5) for Labs D to H using the Jonstrup assay were similar to the estimates observed by the reference standard laboratories, but the span was larger across isolates. There was more consistency in the efficiency estimates for Labs A, B, D (with exception of the Makah isolate), and E. Efficiency estimates (Table 5) for Labs F and H on the Makah isolate were lower than the estimates observed by the reference laboratories. Efficiency estimates for Lab H on the ME03 isolate and estimates for Labs F and H on the FPL2006-005 isolate were higher than the estimates observed by the reference labs.

Table 5. Amplification efficiency (AE) estimates (%). ND: not detected; (–) virus not tested

Method	Isolate ID (genotype)								
Lab	DK5151 (Ia)	1p53 (II)	F13.02.97 (III)	Makah (IVa)	ME03 (IVa)	2000-149 (IVc)	MI03 (IVb)	2004-175 (IVb)	FPL2006-005 (IVb)
Hope et al. (2010)									
A	ND	ND	87	80	89	80	81	85	85
B	ND	ND	96	98	96	97	94	95	102
C	ND	ND	105	87	103	106	94	97	96
Garver et al. (2011)									
A	88	ND	97	91	96	91	94	93	102
B	97	ND	105	100	90	93	90	96	97
C	119	ND	131	110	113	116	106	104	104
Phelps et al. (2012)									
A	107	113	108	106	105	115	110	115	112
B	118	125	121	117	110	113	111	117	111
C	127	155	144	161[a]	172[a]	179[a]	157[a]	153	172[a]
D	–	–	–	124	106	105	–	–	103
E	–	–	–	109	107	111	–	–	109
F	–	–	–	88	102[a]	101	–	–	92
G	–	–	–	86	102	104	–	–	97
H	–	–	–	118	121	111	–	–	117
Jonstrup et al. (2013)									
A	95	91	90	89	94	94	97	93	96
B	95	95	95	93	98	93	92	93	95
C	102	104	109	106	103	103	98	97	104
D	–	–	–	102	98	100	–	–	99
E	–	–	–	98	95	98	–	–	92
F	–	–	–	73	95	101	–	–	100
G	–	–	–	92	103	98	–	–	102
H	–	–	–	80	107	98	–	–	98

[a]A simple linear regression assumes that the relationship between dilution (or concentration) and cycle threshold (Ct) value is constant. The slope represents the expected increase (decrease) in Ct for 1 unit increase in $\log_{10}$ dilution ($\log_{10}$ concentration). It was apparent from the data that the relationship between Ct and $\log_{10}$ dilution ($\log_{10}$ concentration) did not remain constant across all dilutions within the limit of detection (LOD). The observed increase in Ct values between the final 2 dilutions within the LOD was much greater than the observed increase in Ct values for all consecutive dilutions prior. Therefore, the data from the final dilution (corresponding to the least concentrated material) were excluded from the regression analysis that was used to obtain the AE estimates in these instances

Only efficiency estimates for Labs A and B were used as the reference standard for the Phelps assay (Table 5). The efficiency estimates for the Phelps assay were somewhat similar for the Makah and ME03 isolates across laboratories (D–H) with 2 exceptions (Lab D for Makah isolate and Lab H for the ME03 isolate). Efficiency estimates were lower than the reference standard estimates for isolates 2004-175 and FPL2006-005 across laboratories.

Repeatability and reproducibility

The within-plate standard deviation, the between-plate standard deviation, and the total standard deviation for each laboratory were estimated as described earlier using the Jonstrup and the Phelps assays (Table 6). There are no estimates provided for Lab C using the Phelps assay.

First, the standard deviation estimates (within plate, between plates, and total) were compared for Labs A to D where the differences observed are between labs/technicians and not the platform/software. The largest portion of observed variability for both assays is within-plate variability. Lab D (0.14, 0.04, 0.15) performed more consistently (smaller variance estimates) than Labs A (1.34, <0.01, 1.34), B (0.49, 0.07, 0.49), or C (0.89, 0.02, 0.89) using the Jonstrup assay. Similar analyses were completed for Labs E, F, G, and H, where differences observed are due to both differences in lab/technician and differences in the platform/software used. Lab H (0.88, 0.01, 0.88) performed similar to Lab C. Labs E (0.39, 0.10, 0.39), F_1 (0.33, 0.05, 0.34), and G (0.29, 0.04, 0.29) performed most similar to Lab B.

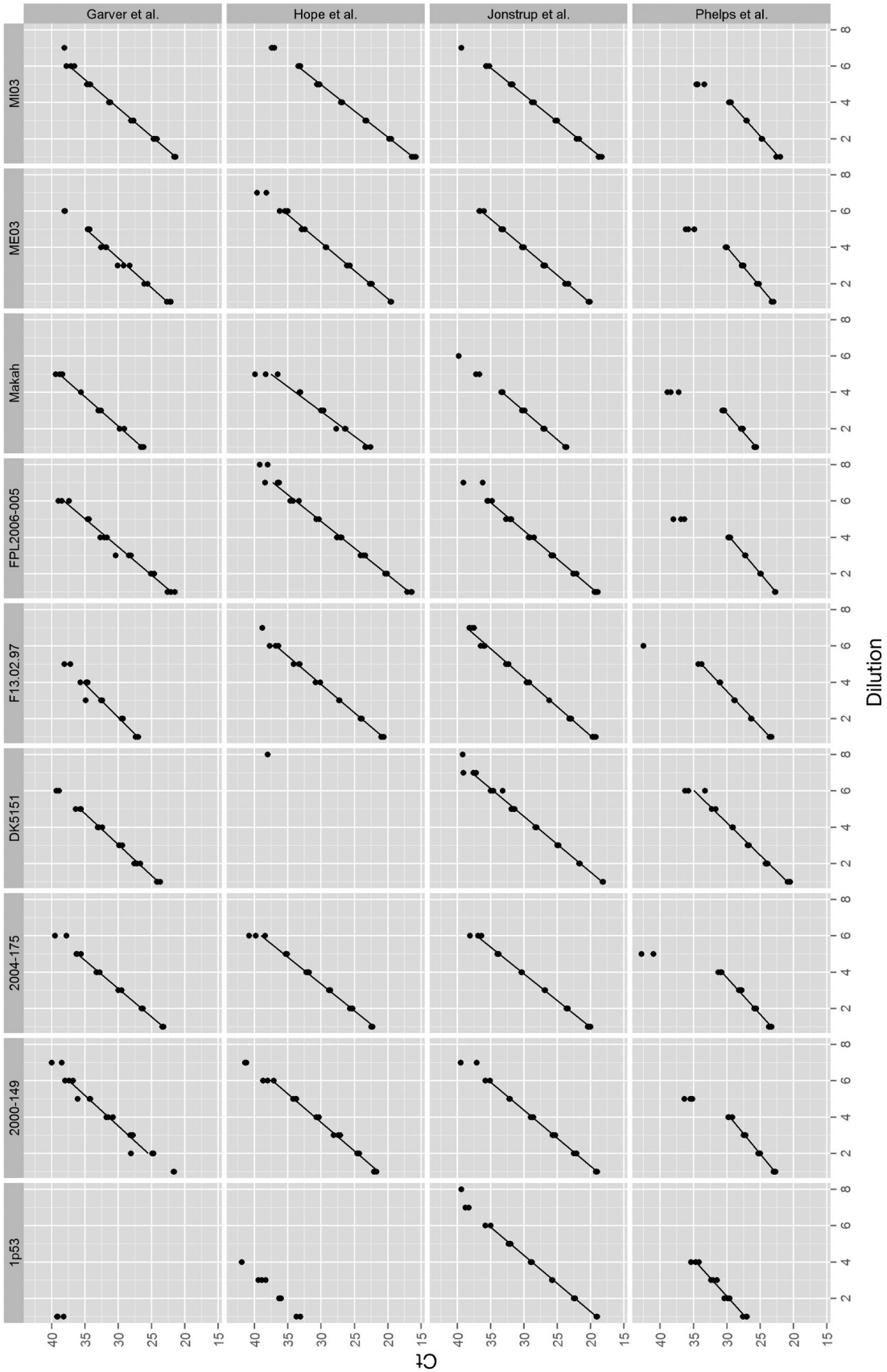

Fig. 1. Phase I limit of detection (threshold cycle, Ct) data plots for Lab C for all assay methods and isolates; x-axis values are not titers but dilution in a 10-fold scheme. Assays tested were those of Garver et al. (2011), Hope et al. (2010), Jonstrup et al (2013), and Phelps et al. (2012)

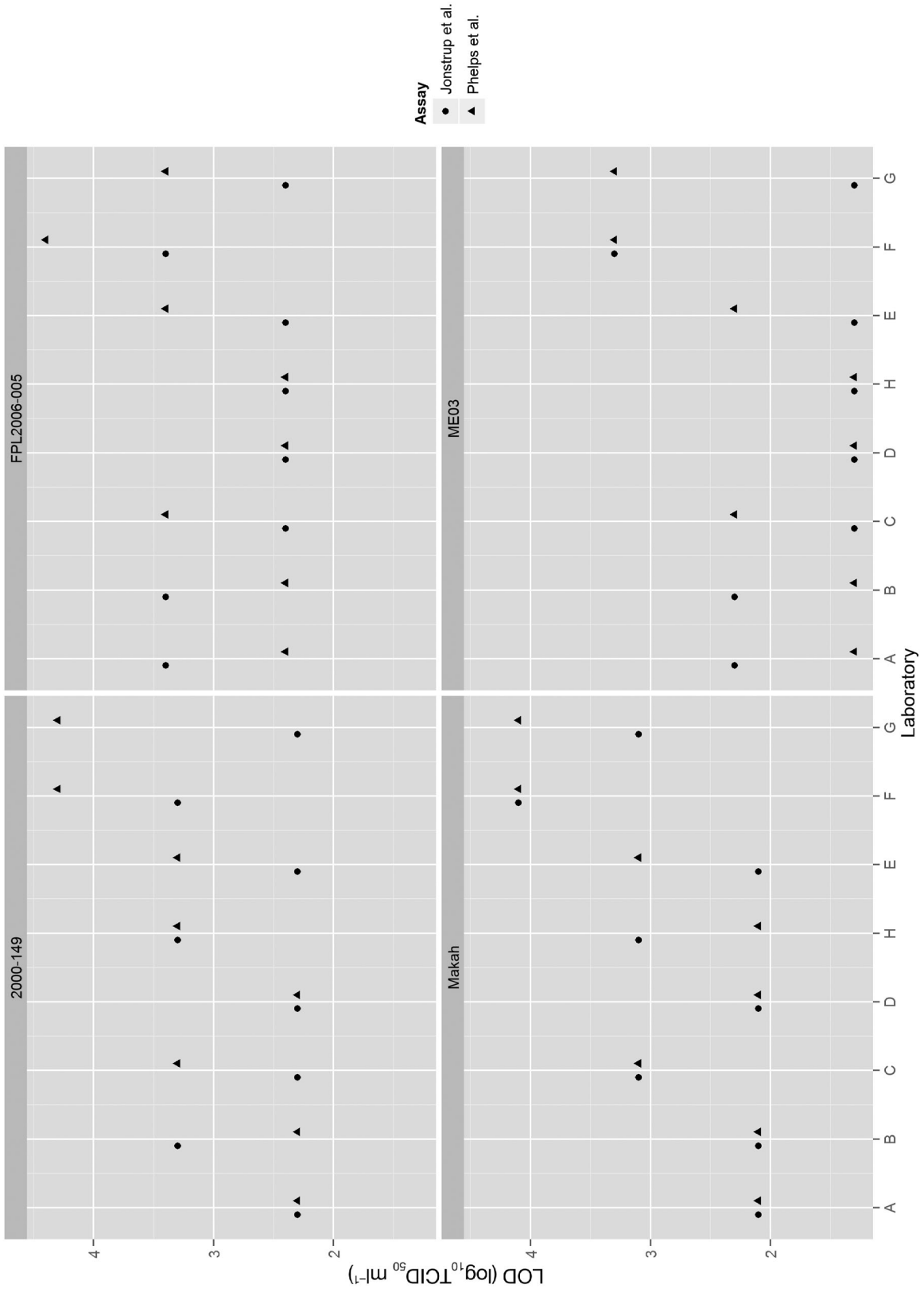

Fig. 2. Comparison of the limit of detection (LOD: the $TCID_{50}$ per ml titer corresponding to the last serial dilution in which all 3 replicates tested positive) for genotype IV isolates of viral hemorrhagic septicemia virus by all laboratories using the assays of Jonstrup et al. (2013) and Phelps et al. (2012)

Table 6. Comparison of assay variation. Within-plate variability = square root of the variance of the random error. Between-plate variability = square root of the sum of the variances of the random plate and random dilution × plate effects. Total variability = square root of the sum of all variance components

Method / Lab	No. of plates	Estimate: Within plate	Between plate	Total SD
Phelps et al. (2012)				
A	5	1.01	0.76	1.27
B	5	0.57	0.56	0.8
D	7	0.43	0.11	0.44
E	8	0.38	0.03	0.39
F_1	2	0.79	1.17	1.41
F_2	6	0.46	0.08	0.47
G	9	0.75	0.06	0.75
H	6	0.26	0.16	0.31
Jonstrup et al. (2013)				
A	5	1.34	<0.01	1.34
B	5	0.49	0.07	0.49
C	5	0.89	0.02	0.89
D	7	0.14	0.04	0.15
E	8	0.39	0.1	0.39
F_1	5	0.33	0.05	0.34
F_2	3	0.16	0.09	0.19
G	9	0.29	0.04	0.29
H	6	0.88	0.01	0.88

Table 7. Efficiency estimates for the viral hemorrhagic septicemia virus (VHSV) MI03 RNA standards provided to the laboratories. The MI03 column shows regression analysis estimates obtained on serial dilutions made in the respective laboratory on the same virus stock as used by the coordinating laboratory to generate the VHSV MI03 RNA standards (positive amplification control). (–) virus not tested

Method / Lab	Plate: MI03	1	2	3	4	5	6	7	8	9
Phelps et al. (2012)										
A	110	149	133	123	133	129	–	–	–	–
B	111	118	119	124	114	117	–	–	–	–
C[a]	157	–	–	–	–	–	–	–	–	–
D		105	102	104	102	103	108	104	–	–
E		125	120	111	128	117	126	143	119	–
F_1		88	119	–	–	–	–	–	–	–
F_2		120	119	126	127	126	124	–	–	–
G		130	132	151	136	108	143	117	131	137
H		112	117	127	132	125	144	–	–	–
Jonstrup et al. (2013)										
A	97	96	96	98	97	101	–	–	–	–
B	92	99	96	99	96	94	–	–	–	–
C	98	94	103	99	98	95	–	–	–	–
D		98	97	98	98	98	96	99	–	–
E		98	97	102	97	98	96	100	98	–
F_1		104	109	102	99	109	–	–	–	–
F_2		98	99	99	–	–	–	–	–	–
G		99	102	104	100	108	99	102	105	100
H		100	98	100	97	99	98	–	–	–

[a]No estimates are provided for Lab C using the assay of Phelps et al. (2012), as the data did not follow the same linear relationship across all plates as one would expect

Lab D (0.43, 0.11, 0.44) performed more consistently than Labs A (1.01, 0.76, 1.27) and B (0.57, 0.56, 0.80) on the Phelps assay. Estimates for total variability using the Phelps assay for Labs E (0.38, 0.03, 0.39), F_2 (0.46, 0.08, 0.47), and H (0.26, 0.16, 0.31) were most similar to Lab B. However, there was a single plate tested by Lab H using both the Jonstrup and the Phelps assays in which the lowest concentration of the RNA standard did not produce a Ct value and had to be excluded in the variability estimates. This should be taken into consideration when assessing overall performance of this laboratory.

Estimates from regression analysis on the data from each laboratory for Ct values observed for the standardized positive amplification RNA controls supplied to laboratories (made from VHSV MI03) using the Jonstrup and the Phelps assays (Table 7) were compared. Estimates of efficiency were closer to 100% for the Jonstrup assay, while all estimates of efficiency for the Phelps assay were >100%. In general, the estimates of efficiency for all laboratories using the Jonstrup assay were similar. However, comparing the spread in values of the efficiency estimates, Labs A (96–101), B (92–99), D (96–99), E (96–102), and H (97–100) demonstrated a more consistent performance than Labs C (94–103), F_1 (99–109), and G (99–108) on the Jonstrup assay. Efficiency estimates using the Phelps assay were not as consistent within a lab or between labs. Lab D (102–108) and Lab B (111–124) performed similarly in terms of spread in values. Lab A (110–149) efficiency estimates had a wider spread in the values than Labs D and B. Labs E, F, G, and H efficiency estimates were more variable and had larger spreads.

Analytical specificity

Genomic RNA from a wide variety of fish and fish pathogens that might be in a surveillance or diagnostic sample were evaluated for cross reactivity (exclusivity) with the primers and probes utilized in either the Jonstrup or the Phelps assay (Table 3). Lab G had 2 instances where Ct values were observed with the Phelps assay. A Ct value of 36.9 was observed for 1 of the 2 replicates of the

high concentration of channel catfish virus (CCV), while both replicates of the low concentration had no observable Ct value. In addition, a Ct value of 31.5 was observed for 1 of the 2 replicates of the low concentration of fathead minnow nidovirus (FHMNV), while both replicates of the high concentration had no Ct values observed. All other pathogens and RNA extracted from fish tissues had no reportable Ct values with either the Jonstrup or the Phelps assays.

DISCUSSION

When making any comparison, some assumptions have to be made, and this was true in our study. First, we assumed that all operators were able to pipette appropriate volumes and were able to make a dilution sequence on a stock of virus. We also assumed (based on previous testing by the coordinating laboratory) that all stocks of viruses (>6 logs) and the PAC set (standardized RNA samples over a 3-log template range) provided to the laboratories were homogenous and that the viral RNA in each vial was not impacted by storage or transport. However, we recognize that differences in laboratory/operator sensitivities may vary among labs (Jonstrup et al. 2013), and hence the selection of a robust assay that meets the testing purpose is paramount for deployment in a laboratory testing network.

Experience with conducting high-throughput testing volumes and maintaining strict quality assurance programs may have contributed to some labs having more consistent performance characteristics as demonstrated in this study (Table 2). Labs A, D, and E are large diagnostic labs and routinely test high volumes of samples by real-time PCR methods for multiple pathogens. In these labs, technicians with a high level of experience conducted the testing. Labs B and H are high-volume diagnostic labs in which the technicians routinely perform conventional PCR assays and were recently trained to perform real-time assays. Labs C and G have dual roles as both diagnostic and research labs with some experience with high throughput testing. Lab F is also involved in both research and diagnostics; here, the technicians were recently trained to perform real-time assays. Experience levels did not appear to impact the consistency of performance (AE estimates for the different VHSV genotype IV representatives or the RNA standard) when using the Jonstrup assay, and this consistency reflects on the robustness of the assay.

Data from 3 labs (C, F, and G) suggest that the Phelps assay may not be as robust and may be sensitive to variation in technical precision. Specifically, the linear regression analysis of the data set for several isolates and the PAC RNA set tested with the Phelps assay in Lab C did not hold a constant linear relationship between the Ct value and the concentration tested. As the same RNA for each isolate or same lot of PAC was utilized in all 4 assays, the failed linear relationships could have resulted from a technical issue during set up of the reactions. Sensitivity to technical precision is also evident in the data set for Lab F, where the assay was conducted by 2 different, less experienced operators in the same laboratory (F_1 and F_2). Lab G had 2 instances that most likely were false positive test results when testing CCV and FHMNV. A comparison of primer and probe sequences used in the Phelps assay with CCV strain Auburn 1, complete genome (GenBank NC_001493), and with the full genome sequence of 6 different isolates of FHMNV (GU002364 and Warg submissions pending) did not reveal a binding site that would generate an amplicon. It is most likely that the false positive results were due to contamination during either the extraction or during set up of the rRT-PCR.

In summary, 8 labs worked collectively to evaluate 4 standardized protocols according to the analytical criteria used to validate an assay as outlined in the OIE guidelines. Three labs established benchmark performance characteristics on a single platform/software for all 4 assays on representative isolates from the 4 genotypes of VHSV. Plots of the regression analysis (data for Labs A and B not shown, Fig. 1 for Lab C) as it relates to the cut-off values utilized in all 4 assays suggest that cutoff values exceeded the linear relationship between the Ct value and $-\log_{10}$ dilution of the virus stock. Cut-off values were established in the original publications and may have been established to offset the fact that in samples where very low copy numbers of template are expected, the distribution of the template within the fish or sample varies, which would be the case in areas where disease is not known to occur. Additional testing would be necessary to determine the number of replicates required for each sample with the current cut-off values. An additional 5 laboratories were used to establish benchmark performance characteristics for the Jonstrup and the Phelps assays with platform and software changes.

In comparing benchmark performance characteristics (analytical sensitivity data) in order to select an assay for surveillance or regulatory purposes, a number of characteristics are used. Most importantly, the assay must detect all strains if the purpose is to declare freedom from a pathogen or to prevent intro-

duction of a pathogen. Second, serial dilutions of reference isolates and inclusion of an RNA standard set provide limit of detection, dynamic range of the assay, and amplification efficiency estimates, all of which help predict the performance of the assay on diagnostic samples within a testing laboratory. Given the precious and limited amount of well characterized diagnostic samples available, it is ideal to determine these characteristics on isolates first.

Analytical performance measures were used collectively to formulate recommendations on the suitability of each of these assays for surveillance and diagnostic purposes pending further evaluation of each assay's diagnostic performance for its intended purpose. Clearly the Hope assay is not suitable for surveillance or diagnostic purposes when attempting to detect all genotypes, as it is not likely that this assay will detect VHSV genotypes I and II and was not considered further. However, this assay has clearly demonstrated to be a useful tool for surveillance of VHSV IVb in the Great Lakes (Bain et al. 2010, Cornwell et al. 2011, 2012). Phase I data established that while the Garver assay had efficiency estimates closer to 100% than the Phelps assay, the Phelps assay performed better in detecting all genotype representatives. The 2-step approach utilized in the Garver assay is generally more suited for diagnostic purposes on a limited number of samples or when sample size is an issue. One-step assays do provide an advantage when surveying large numbers of fish, as they are technically easier to perform, have fewer manipulations, and turnaround time is reduced. The Jonstrup and the Phelps assays are both 1-step assays and would be better suited for large-scale targeted surveillance efforts when testing is conducted in labs with high quality management and work practice systems and were chosen for further study (Warg et al. 2014, this volume).

The data in this study clearly demonstrate that the Jonstrup assay is the most robust of all 4 assays compared. This assay has a broader range of detection than the Phelps assay, and analytical performance characteristics across all platforms, software, and laboratories would predict the ability to detect weak positives better, and hence be more sensitive by this measure.

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