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DEVELOPMENT OF A ONE-STEP DUPLEX RT-qPCR FOR THE QUANTIFICATION OF PHOCINE DISTEMPER VIRUS

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ABSTRACT: Worldwide, stranded marine mammals and the network personnel who respond to marine mammal mortality have provided much of the information regarding marine morbillivirus infections. An assay to determine the amount of virus present in tissue samples would be useful to assist in routine surveying of animal health and for monitoring large-scale die-off events. False negatives from poor-quality samples prevent determination of the true extent of infection, while only small amounts of tissue samples or archived RNA may be available at the time of collection for future retrospective analysis. We developed a one-step duplex real-time reverse transcriptase-quantitative-PCR assay (RT-qPCR) based on Taqman probe technology to quantify phocine distemper virus (PDV) isolated from an outbreak in harbor (*Phoca vitulina concolor*) and gray seals (*Halichoerus grypus*) along the northeast US coast in 2006. The glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene was selected to assess RNA quality. This duplex assay is specific for PDV and sensitive through a range of 10^0 to 10^9 copies ds-plasmid DNA. For the GAPDH target, the reaction in duplex amplified 10^0 to 10^9 copies of ds-plasmid DNA and was detectable in multiple seal species. This assay reduced the likelihood of false negative results due to degradation of tissues and well-to-well variability while providing sensitive and specific detection of PDV, which would be applicable in molecular epidemiologic studies and pathogen detection in field and laboratory investigations involving a variety of seal species.

Key words: Duplex quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR), marine mammal strandings, northeast USA, phocine distemper virus, RNA quality, seal.

INTRODUCTION

In 2006 higher-than-expected mortality in gray seals *(Halichoerus grypus)* and harbor seals (*Phoca vitulina concolor*) in Maine and Massachusetts, US, resulted in the declaration of an unusual mortality event (Matassa et al. 2008). A new North American isolate of phocine distemper virus (PDV USA 2006) was identified during the event, representing the first case of clinical disease and death in seals associated with an isolated strain of PDV in North America (Earle et al. 2011).

Mutations in the genome of PDV USA 2006 are important in understanding some of the changes in pathogenicity observed compared to the 1988 and 2002 PDV European epizootics. Mutations in the F and M genes of PDV USA 2006 were found in virus isolates from brain that were not present in isolates from lung, liver, or blood, suggesting possible virus persistence in the central nervous system (Earle et al. 2011). Phocine distemper virus USA 2006 also has a few amino acid changes in the P, M, and F genes compared to the 1988 European Ulster/Netherlands strain (PDV 1988) responsible for the mass mortality of over half of the harbor seal population in Europe (Harkonen et al. 2006; Earle et al. 2011. Phocine distemper virus USA 2006 is most closely related to the 1988 strain and is believed to have emerged independently from 2002 European strains, allowing multiple lineages to arise and circulate among enzootically infected North American seals (Earle et al. 2011).

Most of what we know about the effects of PDV on seal populations comes from the successful amplification of viral RNA

from tissue samples collected from stranded animals. Determining the amount of virus in tissues from beach cast seals during large-scale die-offs offers several challenges. Tissue degradation in the field and adequate cold storage of samples are common limiting factors (Lipscomb et al. 1996; Wilson et al. 2010). False negatives resulting from poor-quality samples may prevent detecting the true extent of infection. Given these limitations, there is a need for a specific and quality-controlled test that will reliably detect PDV in field-collected tissue samples.

We developed a one-step duplex reverse transcriptase-quantitative-PCR assay (RT-qPCR) assay based on Taqman probe technology to quantify the relative expression of PDV USA 2006 and to assess RNA quality by amplification of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene. The PDV H gene was targeted for its importance in determining host-virus specificity, tropism, and cytopathogenicity (Von Messling et al. 2004, 2006; Ohishi et al. 2008). The GAPDH gene was selected to allow detection of host RNA in a wide variety of seal species. The quantitative duplex RT-qPCR was highly sensitive for both targets and allowed for the detection of false negative samples due to degradation of tissue. This assay represents a significant improvement in the accurate identification of PDV infected seals. The assay can also be used to determine the number of copies of virus relative to infectivity based on 50% tissue culture infectious dose ($TCID_{50}$) calculations, allowing quantitative estimation of virus load in infected tissues.

MATERIALS AND METHODS

RNA extraction

Tissue samples of lung, liver, spleen, and kidney stored at −80 C from harp seal (*Phoca groenlandica*), harbor seal (*Phoca vitulina concolor*), and gray seal (*Halichoerus grypus*) were obtained from the University of New England Marine Animal Rehabilitation and Conservation Center in Biddeford, Maine, USA (UNE MARC), for GAPDH sequencing. Tissue samples from a PDV-negative harp seal (MARC10-003Pg), a PDV-positive harbor seal (MARC06-034Pv), a PDV-negative harbor seal (MAR02-028Pv), and a suspect PDV-positive gray seal (MARC06-019Hg) were obtained for validation of the assay in tissue samples. The initial PDV status of these seals was based on clinical, gross, serologic, and histopathologic findings reported to us by UNE MARC. After tissue homogenization on dry ice and further processed through a Qiashredder column (Qiagen, Valencia, California, USA), we extracted RNA using an RNeasy Plus Mini Kit (Qiagen). The same RNA isolation procedure was used to extract RNA from cultured PDV USA 2006 for PDV H gene sequencing. The RNA quantity (nanograms per microliter) was determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

Virus culture

An isolate of PDV USA 2006 from a harbor seal collected by UNE MARC that died of morbillivirus infection, was kindly provided by Ole Nielsen, Department of Fisheries and Oceans Canada, as well as viruses used for testing assay specificity. A transfected Vero cell line (VeroDogSLAMtag) expressing canine signaling lymphocyte activation molecules (SLAMs) was provided by Y. Yanagi (Nielsen et al. 2008) and used to quantify the $TCID_{50}$ and viral titer for assay sensitivity. VeroDog-SLAMtag cells were cultured and maintained in medium consisting of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (1:1) with L-glutamine and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Gibco, Grand Island, New York, USA), 10% Cosmic Calf Serum (HyClone, Logan, Utah, USA), 200 μg/mL penicillin/streptomycin (Gibco), and 0.25 μg/mL Fungizone (Gibco). Viral isolation medium contained the above with serum reduced to 2%, double the concentration of penicillin/streptomycin (400 μg/mL), and an additional 0.5 μg/mL gentamicin (Gibco).

PDV H gene sequencing

The sequence of PDV USA 2006 was unknown at the beginning of this project. Therefore, the nucleotide sequence of the H gene of the virus was determined by PCR amplification and Sanger dideoxynucleotide chain termination sequencing using the nucleotide sequence of the H gene of DK 2002 PDV (GenBank FJ648456.1) to design forward and reverse primers using Primer3 (Untergasser

et al. 2012). The primers designated H-For and H-Rev were produced by Applied Biosystems (Life Technologies, Grand Island, New York, USA) (Table 1).

The RNA extracted from PDV USA 2006 was reverse transcribed using a Superscript III First Strand Synthesis System for RT-PCR according to manufacturer's instructions (Invitrogen, Carlsbad, California, USA). For amplification of the cDNA, mix 1 was prepared on ice containing 1 μL (10 mM) dNTP, 1 μL H-For Forward primer, 1 μL (10 mM) H-Rev Reverse primer, 3 μL PDV cDNA, and 14 μL H_2O and subsequently combined with mix 2 containing 5 μL 5× buffer A, 5 μL 5% buffer B, and 2 μL Elongase enzyme (Clontech, Mountain View, California, USA) and 18 μL H_2O. The PCR cycling conditions were as described in Table 2a. The PCR products were analyzed using a 1% agarose gel stained with 5 μL Sybersafe DNA gel stain (Invitrogen) using 8 μL PCR product per lane with 2 μL loading dye and 1 kb and 500 kb Gelpilot standards (Qiagen). The gel-isolated product was purified using standard protocols in the Qiaquick gel extraction kit (Qiagen). The band-isolated PDV 2006 H gene PCR product was cloned into a TOPO TA Cloning pCR2.1 plasmid vector (Invitrogen) followed with a Qiaprep Spin Miniprep (Qiagen). Purified DNA product was sequenced using the vector's M13 forward and reverse priming sites (Genewiz, South Plainfield, New Jersey, USA). The derived H gene sequence was compared to the DK2002 and PDV 1988 sequences (GenBank accession FJ648456 and AF479277) and to the published PDV 2006 USA H gene sequence (GenBank accession HQ007902.1).

RNA quality/reference gene sequencing

A published 197-base pair (bp) GAPDH sequence was chosen as an internal control for RNA quality (Grant et al. 2009). The primer sets were designated GRANT GAPDH-F and GRANT GAPDH-R (Table 1). A 197-bp GAPDH ds-DNA standard was created by comparing multiple clones derived from amplification products of harp, harbor, and gray seal RNA derived from multiple liver, lung, and kidney samples from each species. The Qiagen One-Step RT-PCR kit was used with extracted RNA in 25 μL total reaction volumes including 14 μL RNAse-free water, 5 μL 5× buffer, 1 μL (10 mM) dNTPs, 1.5 μL (10 nM) forward primer, 1.5 μL (10 nM) reverse primer, 2 μL Qiagen One-Step RT-PCR enzyme mix, and 1 μL (5–20 ng) RNA. Cycling conditions were as shown in Table 2b. The expected 197-bp amplified product was recovered by band isolation from agarose gel and inserted into a plasmid vector (TOPO TA Cloning pCR2.1 Invitrogen or pGEM-T Easy Vector System; Promega, Madison, Wisconsin, USA). Qiaprep Spin Miniprep (Qiagen) purified plasmid DNA was sequenced using the vector's M13 forward and reverse priming sites (Genewiz). The derived GAPDH sequences were compared to published sequences in GenBank.

Ds-DNA Standards

Double-stranded DNA plasmid standards (ds-DNA) were developed using a 116-bp PDV USA 2006 H gene PCR product and a 197-bp GAPDH PCR product cloned into the plasmid vectors used for sequencing. Copies of the H gene fragment/μL plasmid were calculated using the following equation (Whelan et al. 2003):

$$Copies = \frac{6.02 \times 10^{23}\ (\text{copies/mol}) \times \text{DNA amount (g)}}{\text{DNA length (dp)} \times 660\ (\text{g/mol/dp})} \quad (1)$$

Tenfold dilutions of plasmids were made from 10^9 copies to 1 copy of plasmid per reaction plate for standard curves.

The 116-bp PDV USA 2006 H gene sequence was amplified using primers for PDV 2002 H gene (Table 1). The PCR reaction utilized 34.5 μL H_20, 5 μL 10× buffer (Clontech), 1 μL (10 mM) dNTP (Clontech), 0.5 μL polymerase 50× (Clontech), 2 μL PDV 116 forward primer (10 nM), 2 μL PDV 116 reverse primer (10 nM), and 3 μL cDNA. The PCR cycling conditions for PDV USA 2006 cDNA using 116-bp H gene forward and 116-bp H gene reverse primers were as described in Table 2c. Amplified product was used to create a ds-DNA plasmid by cloning the 116-bp sequence from purified gel product into a plasmid vector (TOPO TA Cloning pCR2.1, Invitrogen). Qiaprep Spin Miniprep (Qiagen) purified DNA product from this plasmid was sequenced using the vector's M13 reverse priming site (Genewiz). The derived 116-bp H gene sequence was compared to the PDV DK2002 sequence, the PDV 1988 sequence, and the PDV USA 2006 sequence (GenBank accession HQ007902.1; Earle et al. 2011).

Development and optimization of primers and probes for duplex RT-qPCR

For singleplex RT-qPCR, each primer and probe set was tested independently using a Quantitect Probe One Step RT-PCR reaction

TABLE 1. Primers and probes used to develop the one-step duplex reverse transcriptase quantitative PCR for phocine distemper virus. Nucleotides preceded with a "+" indicate locked nucleic acid bases.

Primer/probe[a]	Direction	Sequence	nt[b]	Amplicon size (bp)[c]	Temp[d] (C)	Optimal concentration	Primer/probe reference	Oligo synthesis
PDV 2006							Primer 3	
H-For	Forward	5′-AGG GCC CAG GTA GTC CAA CAA TGT TCT-3′	27	1,824	61	400 nM		Applied Biosystems
H-Rev	Reverse	5′-TTT TTT TAT AAT GCT AGA GGT AAA CTG-3′	27	1,824	51	400 nM		Applied Biosystems
PDV H 116 bp[c]							Hammond et al. 2005	
PDV H 116 Forward	Forward	5′-ACC TCG ATG GGC AAT GTG TT-3′	20	116	66.6	300 nM		Sigma-Aldrich
PDV H 116 Reverse	Reverse	5′-GTC TTA CCG TAG ATC CCT TCT GAG AT-3′	26	116	64.6	300 nM		Sigma-Aldrich
PDV H 116 probe	Probe-FAM/TAM	5′-[FAM]CAT GTC CCT CAT ATC AAA ACC TTC GGA GG [TAMRA]-3′	29	–	74	50 nM		Sigma-Aldrich
GAPDH							Grant et al. 2009	
Grant GAPDH-F	Forward	5′-GTC TTC ACT ACC ATG GAG AAG G-3′	22	197	61.5	200 nM		Sigma-Aldrich
Grant GAPDH-R	Reverse	5′-TCA TGG ATG ACC TTG GCC AG-3′	20	197	68.4	200 nM		Sigma-Aldrich
GAPDH probe	Probe-HEX/TAM	5′-[HEX]G[+C]CA[+A]GAGGG [+T]C[+A]T[+C]A[TAMRA]-3′	16	–	64.8	240 nM		Sigma-Aldrich

[a] PDV = phocine distemper virus; GAPDH = glyceraldehyde-3-phosphate-dehydrogenase.

[b] nt = number of nucleotides.

[c] bp = base pairs.

[d] Temp = temperature.

TABLE 2. Cycling conditions for PCR, one-step reverse transcriptase (RT)-PCR, one-step RT-quantitative (q)PCR, and duplex RT-qPCR.

Reaction[a]	Cycling step	Temperature (C)	Time	No. cycles
a. PCR PDV, H gene	Initial denaturation	94	30 s	1
	Denaturing	94	30 s	35
	Annealing	57	30 s	35
	Extension	68	2 min 30 s	1
	Hold	4		
b. PCR PDV, H gene 116-bp amplicon	Initial denaturation	95	2 min	1
	Denaturing	95	30 s	35
	Annealing	57	30 s	35
	Extension	68	30 s	35
	Final extension	68	5 min	1
	Hold	4		
c. One-step RT-PCR, GAPDH	Reverse transcription	50	30 min	1
	Activation	95	15 min	1
	Denature	94	1 min	40
	Annealing	60	45 s	40
	Extension	72	1 min	40
	Final extension	72	10 min	1
	Hold	4		
d. One-step RT-qPCR, GAPDH, and PDV	Reverse transcription	50	30 min	1
	Activation	95	15 min	1
	Denature	94	15 s	40
	Annealing/extension	60	1 min	40
	Hold	4		
e. Duplex RT-qPCR, GAPDH, and PDV	Reverse transcription	50	20 min	1
	Denature	95	5 min	40
	Annealing	95	15 s	40
	Extension	60	32 s	40

[a] PDV = phocine distemper virus; bp = base pair; GAPDH = glyceraldehyde-3-phosphate-dehydrogenase.

mix (Qiagen) on the Applied Biosystems 7500 Real Time Cycler. Targets were run in singleplex in 96-well plates (Table 2d). A minimum of eight log dilutions of each plasmid standard were used as the positive control, as well as a no template control and a reverse transcription negative reaction per sample.

The reaction targeting PDV included 12.5 μL 2× Q buffer, 0.75 μL PDV 116 forward primer (300 nm), 0.75 μL PDV 116 reverse primer (300 nm), 0.5 μL of PDV probe (50 nm), 0.25 μL Q mix, 5.25 μL RNAse-free water, and 5 μL viral culture–derived PDV RNA at 500 ng/μL. Standards for PDV in singleplex were run from 10^0 to 10^9copies/μL. A Taqman probe (FAM/TAM; Sigma-Aldrich, St. Louis Missouri, USA) and primer set previously designed for the PDV DK2002 H gene were optimized and used to detect the 2006 PDV virus (Hammond et al. 2005).

The reaction targeting GAPDH included 12.5 μL 2× Q buffer, 0.5 μL Grant GAPDH-F forward primer (200 nm), 0.5 μL Grant GAPDH-R reverse primer (200 nm), 2.4 μL of 1:40 GAPDH probe (240 nm), 0.25 μL Q mix, 3.85 μL RNAse-free water, and 5 μL RNA derived from seal tissues at 500 ng/μL. Standards for GAPDH in singleplex were run from 10^0 to 10^9copies/μL. The fluorescent reporter dye was modified from VIC/TAM to HEX/TAM (Sigma-Aldrich) (Grant et al. 2009).

The PDV 2006 and GAPDH primers and probes were optimized for a duplex reaction using Qiagen Quantifast Multiplex RT-PCR +R in 96-well plates. Two initial reactions, using 100 ng/μL PDV RNA derived from Vero cell culture and separately using 100 ng/μL seal-derived positive PDV tissue sample, were first assayed and successfully confirmed the

detection of PDV and cell culture GAPDH, as well as PDV and seal-derived GAPDH. For the optimization of this assay, copies were determined using a 100 ng/μL seal-derived tissue RNA in duplicate. Controls were plated as in singleplex. Each 20 μL sample reaction contained: 10 μL Quantifast Master mix, 0.4 μL ROX, 0.6 μL PDV forward primer (300 nm), 0.6 μL PDV reverse primer (300 nm), 0.4 μL PDV probe (50 nm), 0.4 μL GAPDH forward primer (200 nm), 0.4 μL GAPDH reverse primer (200 nm), 1.92 μL GAPDH probe (240 nm), 0.2 μL RT mix, and up to 5 μL of RNA with sterile RNAse-free water. Concentrated seal tissue–derived RNA was first diluted to 100 ng/μL with sterile PCR water. Standards were run under the same conditions with additional 3.08 μL water and 1 μL of each of the PDV and GAPDH standard ds-DNA plasmid in 10-fold serial dilutions from 10^0 to 10^9 (Table 2e.) Each fluorescent reporter signal was measured against ROX added to the reaction mixture above. The lowest level of detection was the lowest dilution of the standard to amplify. The baseline cycle threshold value was set at the exponential increase phase of the reaction for each standard.

Precision and efficiency of the singleplex and duplex assay

Precision was calculated based on the R^2 of the standard curve. The PDV standards were diluted from ds-DNA plasmid standards of 7.89×10^9 copies/μL, and GAPDH standards were diluted from plasmid standards of 8.89×10^9 copies/μL. The PCR efficiencies were calculated for each singleplex reaction and for each channel in a duplex reaction. Percentage efficiency was calculated by the following formula (Rasmussen 2001):

$$\text{E (PCR efficiency)} = \left(10^{(1/\text{slope})} - 1\right) \times 100\% \qquad (2)$$

Based on this equation, a PCR efficiency of a 100% is equal to a slope of −3.32.

Repeatability and reproducibility of the singleplex and duplex assay

The coefficient of variability (CV; CV=standard deviation/mean) was used to calculate the intra-assay variability. Three plasmid standards (10^3, 10^5, 10^7) in eight replicates within a single experiment for the singleplex assay and three replicates for the duplex assay were measured. Interassay variability CVs were measured from three independent experiments using the same three standards quantified in the intra-assay variability measurements.

Specificity of the singleplex and duplex assay

Specificities of the singleplex and duplex assay were performed using virus cultures. A QiaAmp Viral RNA Mini extraction kit (Qiagen) was used to isolate RNA from viruses in culture, including dolphin morbillivirus (DMV), Steller sea lion reovirus (Palacios et al. 2011), canine distemper virus (CDV) Lederle strain, cetacean morbillivirus (CeMV) isolated from *Tursiops truncatus* 2013, PDV (Osterhauns 1988), and a seal picornavirus (Kapoor et al. 2008). Viral RNA aliquots were diluted 1:10 in sterile PCR water. Quantity of RNA (ng/μL) was assessed using a Nanodrop 1000 Spectrophotometer (Thermo Scientific) and 100 ng/μL of viral RNA was used in the duplex and singleplex assay as described earlier.

Relative infectious potential (TCID50) of PDV USA 2006

The $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$) of PDV USA 2006 was calculated in duplicate using 10-fold dilutions of virus grown on SLAM-transfected Vero cells. Results were analyzed using the Spearmann-Karber Titre calculator. The known $TCID_{50}$ stock virus was made into 10-fold dilutions, and RNA was then extracted using the Qiagen Mini Viral RNA Isolation Kit. We used PDV RNA in duplex RT-qPCR as described earlier. The $TCID_{50}$ equivalents were determined from the copy numbers of ds-DNA standards and therefore infectious PDV equivalents in duplex reaction samples.

RESULTS

A subset of the resulting bands from RT-PCR, singleplex, and duplex RT-qPCR reactions were sequenced and indicated specificity of the PDV H gene (1824 bp), GAPDH (197 bp), and PDV (116 bp) sequences. Comparison of the H gene from three clones derived from first passage of PDV 2006 USA indicates three differences when compared with the previously sequenced 1952-bp PDV DK2002 (GenBank accession FJ648456.1) within the 116-bp sequence detected by the primers used for this assay (Fig. 1). Two of these differences correspond to changes previously described in the 1824-bp 2006

PDV2006 Plasmid sequence @ nt 597

597	ACCTCGATGG	GCAATGTGTT	C**T**CGTTAGCT	GTTTCCTTGT
637	CCATGTCCCT	CATATCAAAA	CCTTCGGAGG	TAATTA**A**CAT
677	GTTGAC**T**GCA	ATCTCAGAAG	GGATCTACGG	TAAGAC

T S M G N V F **S** L A V S L S M S L I S K P S E V I **N** M L T A I S E G I Y G K

PDVUSA2006 (sequence Earle et al. 2011) @ nt 597

597	ACCTCGATGG	GCAATGTGTT	C**T**CGTTAGCT	GTTTCCTTGT
637	CCATGTCCCT	CATATCAAAA	CCTTCGGAGG	TAATTA**C**CAT
677	GTTGAC**T**GCA	ATCTCAGAAG	GGATCTACGG	TAAGAC

T S M G N V F **S** L A V S L S M S L I S K P S E V I **T** M L T A I S E G I Y G K

PDV2002DK Sequence @ nt 597

597	ACCTCGATGG	GCAATGTGTT	C**C**CGTTAGCT	GTTTCCTTGT
637	CCATGTCCCT	CATATCAAAA	CCTTCGGAGG	TAATTA**A**CAT
677	GTTGAC**A**GCA	ATCTCAGAAG	GGATCTACGG	TAAGAC

T S M G N V F **P** L A V S L S M S L I S K P S E V I **N** M L T A I S E G I Y G K

FIGURE 1. Sequence comparison of the phocine distemper virus (PDV) H gene from strains PDV USA 2006 sequenced in this study, PDV USA 2006 (GenBank accession HQ007902.1) and PDVDK2002 (GenBank accession FJ648456.1) between nucleotide 597 and 712. Bold underlined nucleotides indicate differences between sequences. Nucleic acids underlined with a zig-zag line indicate silent mutations. Below nucleotide sequences are corresponding in amino acid sequences with changes underlined.

sequence (GenBank accession HQ007902.1, Earle et al. 2011) and one does not. In the DK2002 sequence, at amino acid 200, there is a substitution from P (proline) to S (serine). This change is seen in our sequence as well as in the recent 2006 PDV virus sequence of the H gene corresponding with a silent base pair substitution at base 618, from nucleotides T to C. There is also a silent mutation at base 683 with the substitution of nucleotides A to T in both 2006 sequences when compared with the DK2002 sequence. The differences between the two PDV 2006 USA sequences lie at amino acid 218 at base 673 and results in an amino acid substitution of N (asparagine) to T (threonine). The probe used in the development of this assay utilizes a region conserved in the 1988, 2002, and 2006 isolates (Fig. 2) (Hammond et al. 2005).

The 197-bp GAPDH plasmid sequences derived from harbor, gray, and harp seal tissues were compared to the previously published sequences and to GAPDH sequences in GenBank (Grant et al. 2009). At least three clones from three individuals of each species, derived from lung, liver, or kidney, were sequenced. Differences exist within the probe region at positions 54 and 55 of the 197-bp sequence (Fig. 3). All plasmids of the three species amplified

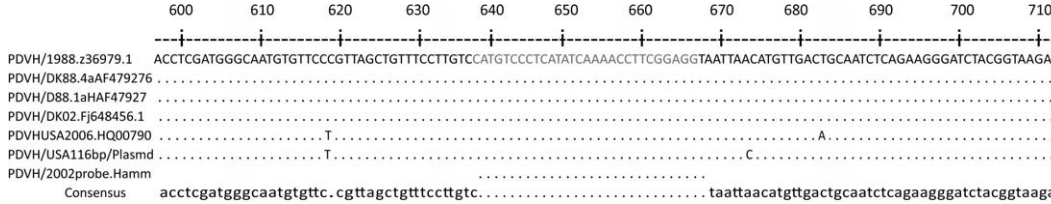

FIGURE 2. Alignment of phocine distemper virus (PDV) H gene sequences and targeted PDV H gene plasmid and probe sequence used in this study. Sequences of PDV H gene are denoted by corresponding GenBank accession numbers. Gray denotes areas of high consensus.

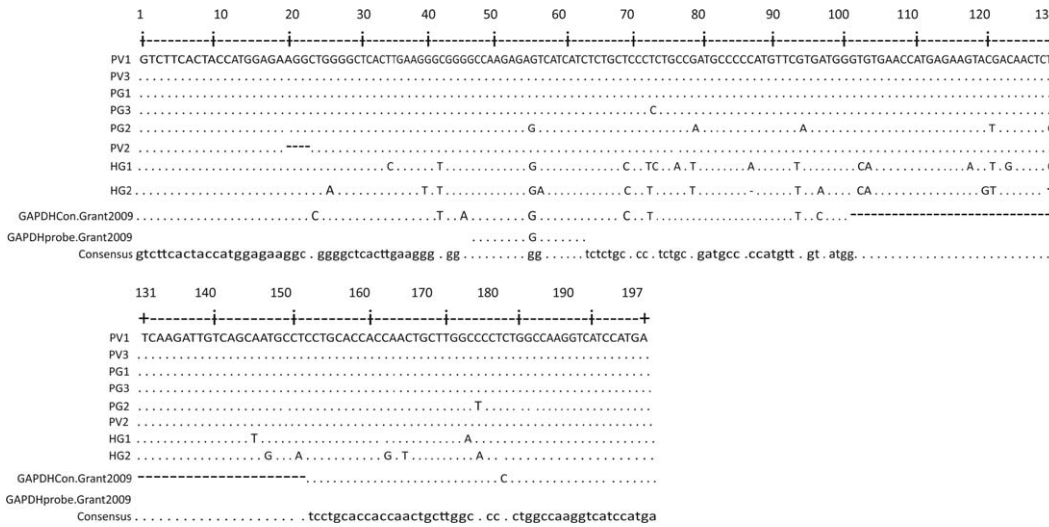

FIGURE 3. Sequence alignment of 197-base-pair glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene fragments from harbor seal (*Phoca vitulina concolor* [PV]), gray seal (*Halichoerus grypus* [HG]), and harp seal (*Phoca groenlandica* [PG]). Sequences are aligned to the consensus sequence and GAPDH probe used in the development of our assay. The plasmid probe used for the development of the assay was derived from harbor seal lung tissue, represented as PV1.

using the GAPDH primer and probe set despite the mismatches. The final GAPDH plasmid standard was derived from harbor seal lung tissue.

The previously published primer and probe sequence used to amplify 2002 PDV was used in a singleplex reaction to compare with the duplex reaction. The singleplex PDV real-time RT-PCR reaction amplified PDV ds-plasmid DNA standards, detecting 10^1 to10^9 copies with an R^2 of 0.9849, a slope of −2.88, and an efficiency of 121.94%. The singleplex GAPDH real-time RT-PCR reaction amplified ds-plasmid DNA in triplicate, detecting 10^2 to10^8 copies with an R^2 of 0.9815, a slope of −3.3223, and percentage efficiency of 99.984%.

Intra-assay variability for the singleplex PDV assay for the three plasmid standards (10^3, 10^5, 10^7) were 1.58%, 5.6%, 5.54% and 4.85%, 7.05%, 4.44% for the singleplex PDV and GAPDH assays, respectively. Interassay variability for the same standard dilutions for PDV were 5.89%, 7.86%, 9.91% for PDV and 5.36%, 1.89%, 6.81% for GAPDH.

The duplex real-time RT-PCR experiments amplified PDV ds-plasmid DNA from 10^0 to 10^9 copies with an R^2 of 0.981, a slope of −3.15897, and an efficiency of 107.28%. For the GAPDH ds-plasmid DNA, the reaction amplified 10^0 to 10^9 copies with an R^2 of 0.9865, a slope of −2.998, and an efficiency of 115.55%.

With three randomly selected replicates of the duplex assay, the sensitivity of PDV ds-plasmid DNA detection ranged from 10^2 to 10^9 copies with an R^2 of 0.9781. With the lower detection limit of 10^1 to 10^9 copies, the R^2 diminished to 0.9682. The sensitivity of the GAPDH detection in the same three replicates ranged from 10^1 to 10^9 copies with an R^2 of 0.9733. With the lower detection limit of 10^0 to 10^9, the R^2 diminished to 0.9654.

Intra-assay CVs for the duplex assay for the three plasmid standards (10^3, 10^5, 10^7) were 2.73%, 1.38%, 3.69% and 2.24%, 1.33%, 3.14% for PDV and GAPDH, respectively. Interassay CVs for three separate duplex assays for PDV were 4.75%, 16.84%, and 9.03%. The interassay CVs for GAPDH were 2.4%, 5.47%, and 4.76%.

Both singleplex and duplex assays were determined to be highly specific for PDV. No detection occurred with DMV, CeMV, CDV, Steller sea lion reovirus, or seal picornavirus (data not shown).

PDV was calculated to have a $TCID_{50}$ of 4.4 ($log10TICD_{50}$/mL) equivalent to 25,118 infectious particles per mL with a limit of detection and sensitivity of the assay of 2.5 infectious particles, equivalent to (mean±SD) 6.25±4.3 copies of PDV standard, to 25,118 infectious particles with an equivalent of 479,656±419,797 copies PDV standard.

Using this assay on lung, liver, kidney, or spleen tissues from seals of suspect or known PDV status, PDV was detected in kidney of the known positive harbor seal ($36{,}641\times10^4$ copies PDV standard/μL RNA; 2.12×10^6 copies GAPDH standard/μL RNA). No PDV was detected in the PDV-negative harbor seal in lung, liver, spleen, or kidney (9.43×10^5, 3.5×10^8; 83, 9.5×10^6 copies GAPDH standard/μL RNA). No PDV was detected in the lung of a PDV-negative harp seal (156.6 copies GAPDH standard/μL RNA), and neither PDV nor GAPDH was detected in the spleen sample of the same individual, resulting in the identification of a possible false negative sample. No PDV was detected in the suspect positive gray seal in lung, liver, spleen, or kidney (226, 8.93×10^4, 2.95×10^6, 3.15×10^5 GAPDH/μL RNA).

DISCUSSION

Our assay assessed RNA sample quality by measuring GAPDH, while simultaneously quantifying the target virus, PDV. The use of an endogenous reference gene to assess RNA quality has previously been used in RT-PCR and RT-qPCR studies (Inderwies et al. 2003; Hammond et al. 2005; Persson et al. 2005; Grant et al. 2009; Takano et al. 2010; Brisco and Morley 2012). The primer and probe set we used has been shown to amplify 14 marine mammal species in singleplex RT-qPCR (Grant et al. 2009). The duplex assay offers benefits over using one- and two-step RT-qPCR methods including the reduction of template RNA necessary, reduced chance for contamination over two-step assays, and simultaneous assessment of sample quality. Interpretation of the results is facilitated in that the target PDV gene is quantified in the same reaction as the endogenous control gene.

Interpreting RT-qPCR results from samples collected from stranded and often decomposing carcasses is essential in appropriately and unequivocally diagnosing cases of infection, while avoiding false negative findings. Despite that viral nucleic acid may be protected from degradation compared to host nucleic acid, a means to assess interpretable samples as a whole is necessary. Low RNA integrity can be assessed through a multiplexed endogenous control gene. The use of an endogenous locked nucleic acid LNA primer and probe set in duplex, validated by sequencing in an additional two species of seal, will be useful in detecting false negative PDV samples (Fleige and Pfaffl 2006; Hall et al. 2006).

Previously published serial dilution of ds-DNA plasmid standards based on the N gene of PDV using a singleplex RT-qPCR recorded a sensitivity of 10^1 (Grant 2008). Using a ds-DNA plasmid standard, detection was reported to be 10-fold more sensitive for PDV over RNA standards allowing for smaller amounts of detectable RNA (Grant 2008). The singleplex assay we used for the H gene amplicon is equally as sensitive as those previously reported. The duplex assay gave greater sensitivity measuring one copy of PDV ds-DNA standard. The GAPDH gene in singleplex detected 10^2 copies while the duplex assay was able to detect GAPDH to 10^1 with high precision. The variability between assays was higher in duplex, illustrating the importance of running standards in each assay.

While the sensitivity of RT-qPCR is one of the most useful benefits of the assay, it does not provide biological relevance as to

the infectious nature of the virus. Calculating the corresponding $TCID_{50}$ of the virus stock allows for more relevant quantification of the viral load. The sensitivity of the $TCID_{50}$ assay not only relates to copies of standard but to the number of infectious particles detectable. Measuring PDV through the duplex assay is therefore more sensitive than quantifying virus through viral titers. Furthermore, using the $TCID_{50}$ equivalents gives the value of viral copy number biological relevance.

Optimizing multiplex reactions offers several challenges. Accurate quantification is dependent on optimization of all components of the reaction mixture to address unequal rates of gene expression and RNA quantity of the target genes (Persson et al. 2005). While the PDV and GAPDH singleplex reactions had excellent precision, there was a slight loss of precision and PCR efficiency in duplex. Efficiency over 110% could suggest possible overamplification of the more abundant target in the duplex reaction or a decrease in the theoretical doubling of DNA in reaction amplification at the far ends of the range of detection (Bio-Rad Laboratories 2006; Applied Biosystems 2007; Karlen et al. 2007). The kinetics related to the amplification of plasmid-derived RNA also differ from tissue-derived RNA as well as ds-DNA plasmid standards (Plaffl 2004). In addition, the duplex reaction may favor the LNA based probe and primer set. Designing an LNA primer and probe for the PDV H gene target could increase efficiency without reducing the precision (R^2). A recent study targeting cetacean morbilliviruses in singleplex RT-qPCR using the Universal Probe Library could similarly be used for developing an H gene probe for PDV with higher efficiency in multiplex (Rubio-Guerri et al. 2013). Future development and optimization of an exogenous RNA control would also aid in a more robust multiplex assay.

Determining the identity and quantity of infectious disease agents in stranded marine mammals presents several unique challenges. Degradation of RNA is an inevitable limitation of diagnostic testing in marine mammal mortality events and continues to be a challenge even with increasingly sensitive molecular methods (Stroud and Roffe 1979; Krafft et al. 1995; Saliki et al. 2002). This assay helps address some of these challenges by providing a useful method to eliminate false negative samples from a dataset due to degradation of RNA of tissues and providing sensitive levels of detection of PDV. The sensitivity of this assay may also provide a useful method to assess risk of exposure from subclinical carriers of the virus, thereby enhancing the ability to manage and prevent viral spread in rehabilitation facilities. The duplex assay may also be used to quantify viral load during in-vitro infection experiments. This assay can therefore provide a useful means to investigate the pathogenicity and epidemiology of PDV in field and laboratory settings to improve our understanding of the ecologic impact of PDV.

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