A novel quantitative real-time PCR diagnostic assay for fecal and nasal swab detection of an otariid lungworm, \textit{Parafilaroides decorus}

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\textbf{A B S T R A C T}

\textit{Parafilaroides decorus}, also known as sea lion lungworm, is a metastrongyloid nematode that infects otariid hosts, such as the charismatic California sea lion, \textit{Zalophus californianus}. \textit{P. decorus} causes bronchoheptalstitial pneumonia, respiratory distress, reduced ability to swim, dive and hunt and as a result, increased mortality particularly in young animals. Respiratory disease is a leading cause of stranding and admission to rehabilitation centers on the Pacific coast. Low-coverage genomic sequencing of four \textit{P. decorus} individuals analyzed through Galaxy’s RepeatExplorer identified a novel repeat DNA family we employed to design a sensitive quantitative PCR (\textit{qPCR}) assay for diagnosing infections from fecal or sputum samples. The assay detects as little as 10 fg of \textit{P. decorus} DNA and a linear regression model developed using a standard curve can be used to estimate the concentration of \textit{P. decorus} DNA in a sample, $\pm 0.015$ ng. This knowledge can be leveraged to estimate the level of parasite burden, which can be used to design improved treatments for animals in rehabilitation. Improved treatment of infections will aid in more animals being successfully released back into the wild.

\begin{section}{1. Introduction}

To the detriment of much marine life, global warming is predicted to increase the range, prevalence, and virulence of aquatic parasites (Harvell et al., 2002). Accurate identification of these parasites can allow for better assessment and treatment of pathogenic threats to wild marine mammals and treat infections of those being cared for in rehabilitation facilities. Our goal is to create sensitive, species-specific assays for diagnosis of pre-patent infection in California sea lion (\textit{Zalophus californianus}) patients who present with respiratory disease and to assist clinicians in monitoring the efficacy of anthelmintic treatment (Field et al., 2018). Current diagnostic practice is to use a Baermann test to detect larvae in feces, but the test can only detect a patent infection and it lacks sensitivity and specificity and is not quantitative (Pilotte et al., 2016, 2019; Easton et al., 2017). While PCR has been repeatedly demonstrated as superior in both sensitivity and specificity, the use of traditional ribosomal or mitochondrial DNA targets can also be insufficiently sensitive in detecting trace amounts of DNA from parasite eggs or larvae in feces (Pilotte et al., 2019). By using a repeat-based quantitative PCR (\textit{qPCR}) assay with a higher number of genome targets, assays are not only more sensitive but allow for the estimation of parasite burden (Pilotte et al., 2019). In nematodes, these non-coding repeats evolve quickly enough that they tend to be species-specific (Pilotte et al., 2016). \textit{Parafilaroides decorus}, a common lungworm of otariids, is a metastrongyloid nematode. The adults are found in the bronchioles and alveolar parenchyma of sea lions of all ages but are particularly prevalent in pups and juveniles (Greig et al., 2005). In most animals \textit{P. decorus} causes minimal inflammation and only mild clinical signs such as coughing as larvae are expelled from the airway (Measures, 2018). However, heavy parasite burdens in young animals are characterized by bronchitis, bronchiolitis and interstitial granulomatous pneumonia, which reduce the ability to swim, dive, and hunt, and result in increased mortality in these young animals. Bronchioles are often obstructed by nematodes causing inflammatory exudate and thick mucus. Infection is often complicated by opportunistic bacterial resulting in further pulmonary consolidation and often abscessation. The result can be severe dyspnea leading to stranding or death (Measures, 2018). In addition to pulmonary pathology, the life cycle of the parasite involves migration of larvae through the vascular system to the lung potentially causing fatal pulmonary endarteritis, thrombosis and infarction (Dailey, 1970; Seguel et al., 2018). While the primary host, the California sea lion (\textit{Zalophus californianus}), has been rated

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P. decorus are ovoviviparous shedding first stage larvae into the lower airways where they can be observed in mucus droplets attached to the epithelium, and then moved by the mucus escalator to the pharynx, swallowed and shed in feces (Dailey, 1970; Measures, 2018). Either sputum or feces could be used for detection of larvae but the fecal Baermann test is the standard method (Measures, 2018). However, Baermann tests and microscopy are time-consuming, insensitive, and not species-specific (Pilotte et al., 2016). By using a species-specific qPCR assay, we can use the cycle at which a sample reaches exponential amplification, or the quantification cycle (Cq value), to estimate the amount of DNA from the targeted species in the sample. In this way, not only can the presence/absence of a particular parasite be determined from a sputum or fecal sample, but the infection intensity can also be estimated. To make such estimates, a predictive model must first be constructed. By isolating pure genomic DNA from a control P. decorus sample and using the assay to test a serial dilution of this DNA, we can obtain the data necessary to create a linear regression model of the relationship between input P. decorus DNA and the Cq value for this particular assay.

Parasitic burden can be inferred from the estimated DNA concentration in the feces or sputum. Armed with knowledge of both the species causing the infection and the burden of infection, marine mammal clinicians could choose the most appropriate therapeutic regimen for a particular patient and monitor the efficacy of anthelmintic treatment with sequential tests. These animals, if successfully treated, can then be released back into the wild, infection-free.

2. Materials and methods

2.1. Parasite, fecal, and sputum sample acquisition

Parafilarioidea decorus and other nematode parasites were obtained from stranded, deceased otariids, while fecal and sputum samples were obtained from rehabilitating otariids collected by The Marine Mammal Center (TMMC, Sausalito, CA; Table 1). All host otariids were stranded on the Pacific coast of California. Each identification number represents the single host animal from which the nematode specimen was collected (Table 1). Negative control fecal and sputum samples were collected by the New England Aquarium (NEAQ, Boston, MA) and the Long Island Aquarium (LIA, Long Island, NY), respectively (Table 1). No parasites were obtained from live animals, and no live animals were harmed in the acquisition of samples for this study. All materials were obtained with a permit from the National Oceanic and Atmospheric Administration (NOAA) authorized under the regulations at 50 CFR 216.22(c)(5) and 216.37 of the Marine Mammal Protection Act, which states that marine mammal ‘parts’ can be transferred for scientific research purposes. All materials were collected in accordance with the NOAA regulations. All parasites were transferred from the rehabilitation facility to Smith College in 70–100% ethanol on ice. Fecal and sputum samples were transferred on ice without storage media. Upon acquisition, all samples were placed at −80 °C for long-term storage.

2.2. Parasite, fecal, and sputum sample DNA extractions

Total genomic DNA from whole nematode samples were isolated following established protocols, using organic phenol-chloroform extraction and ethanol precipitation (Keroack et al., 2016). DNA from fecal samples was isolated using the Qiagen DNeasy PowerSoil Kit (Hilden, Germany) following the manufacturer's protocols using a 0.5 g sample of feces. DNA was extracted from sputum (collected with nasal swabs) using the Qiagen DNeasy Blood & Tissue Kit (Hilden, Germany) following the manufacturer's protocols using a 0.5 g sample of sputum.

Purity of the DNA was checked using a Thermo Fisher Scientific nanodrop spectrophotometer (Waltham, MA, USA) and concentration was determined using a Thermo Fisher Scientific Qubit® 2.0 Fluorometer (Waltham, MA, USA) using 2 μl of the DNA sample with the Invitrogen dsDNA HS Assay Kit (Waltham, MA, USA).

2.3. Parasite identity confirmation by molecular barcoding

The internal transcribed spacer region 2 (ITS2) of whole nematode isolates was Sanger sequenced to confirm morphological species identifications (Supplementary Table 1). ITS2 was amplified using previously published nematode-specific primers, with 5′-AGTTGGAATTTCACAGGCGATTGAG-3′ as the forward primer and 5′-AGCGGGTAAATCAGACTGAGTGTGA-3′ as the reverse primer (Rishniw et al., 2006). Amplification was done using Thermo Fisher Scientific’s Phusion High-Fidelity PCR Kit (Waltham, MA, USA) under the following conditions: 98 °C for 3 min as an initial denaturing step; followed by 35 cycles of 98 °C for 30 s for denaturing, 60 °C for 30 s for annealing, and 72 °C for 1 min for extension; and a final extension of 72 °C for 10 min. PCR products were Sanger sequenced using Thermo Fisher Scientific’s BigDye™ Terminator v3.1 Cycle Sequencing Kit (Waltham, MA, USA) following the manufacturer's protocol. Sequences were compared to the GenBank database using NCBI's BLASTn to confirm the species identity of the nematode samples (Altschul et al., 1990; Sayers et al., 2019).

2.4. Low-coverage genome sequencing (illumina)

Four P. decorus samples were pooled for low-coverage genome sequencing from hosts GFS-151, CSL-13271, CSL-13295, and CSL-13301 (Table 1). Genomic libraries for next-generation sequencing on Illumina's MiSeq System (San Diego, CA, USA) were fragmented using NEBNext® dsDNA Fragmentase® (Ipswich, MA, USA) following the manufacturer's protocol. The fragmentase reaction was incubated for 19 min to achieve a fragment profile averaging 550 base pairs. The sample size profile was confirmed using Agilent Technologies’ Agilent 2100 Bioanalyzer with the Agilent Bioanalyzer High Sensitivity DNA Kit (Santa Clara, CA, USA) before continuing with the library preparation to ensure the desired fragment size was obtained.

Fragmented total genomic DNA was then modified using the NEBNext® DNA Library Prep Master Mix Set for Illumina® (San Diego, CA, USA) according to manufacturer's protocols with NEBNext® Multiplex Oligos for Illumina® (Ipswich, MA, USA) for indexing. Total input DNA for library construction was 540 ng. Library quality was validated using the Agilent Bioanalyzer High Sensitivity DNA kit (Santa Clara, CA, USA) and was sequenced using MiSeq Reagent Kit V3 and a 1% PhiX control (San Diego, CA, USA). The resulting sequences were converted into FASTQ files and had their adaptor and index sequences removed using BaseSpace® (basespace.illumina.com). Genious 9.1.8 (https://www.geneious.com) was used to convert the sequences from FASTQ to FASTA files and to merge the paired reads using the FLASH plugin (Fast Length Adjustment of SHort reads, version 1.2.9, Magoc and Salzberg, 2011). The resulting sequence data can be found on NCBI’s Sequence Read Archive (SRR11068184).

2.5. Repeat cluster generation

P. decorus genomic repeat family selection was done following an established, previously published workflow using standard parameters for all tools (Grant et al., 2019; Pilotte et al., 2019; Keroack et al., 2018; Papaiakovou et al., 2017; Pilotte et al., 2016; Novák et al., 2010, 2013). Unlike the workflows used in previous studies that only used sequences from one species to generate genomic repeat clusters, sequences from three outgroup species were included so that repeat family homology would be identified across the species, and repeat families present in other closely related species could be avoided in designing the diagnostic assay to ensure species-specificity. The closest
species included in the sequencing was another pinniped-infecting lungworm in the metastrongylid group, *Ostrostrongylus circumitus* (Chilton et al., 2006). Additionally, a non-metastrongylid strongylid was included, *Necator americanus*, as well as a non-strongylid nematode as the furthest outgroup, *Acanthochelonia spiracauda* (Chilton et al., 2006). The samples included for the repeat cluster analysis and their sources are described in Supplementary Table 2. No low-coverage genomic data from a *Parafilearoides* species that was not *P. decorus* were available to use as an outgroup within the same genus.

Raw FASTA files from each species were uploaded to the Galaxy RepeatExplorer server (http://www.repeatexplorer.org/) (Novák et al., 2015). Once the FASTA files were uploaded to RepeatExplorer, files larger than one million reads were randomly subsampled down to one million reads to save computing time. Once subsampled as needed, the samples were head-to-tail concatenated to create one FASTA file containing sequences from *P. decorus* and the three outgroup species. Genomic repeat families were then identified using the RepeatExplorer Clustering tool on the concatenated FASTA sequences. The default settings were retained except the cluster size threshold for detailed analysis was lowered from 0.01% to 0.001% to maximize output.

### 2.6. Selecting a *P. decorus* repeat DNA family for qPCR assay design

For each of the 104 repeat families discovered, the number of sequencing reads from *P. decorus* and each of the three outgroups that matched the repeat family were tallied. The number of reads from each species belonging to each of the repeat families is summarized in Fig. 1. For the diagnostic assay, a repeat family with zero reads in the three outgroups were selected as candidates. These candidates were then analyzed using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure that the candidate repeat families were not ribosomal or mitochondrial DNA sequences, or a close match to DNA sequences found in other related parasite species, the marine mammal host or human (in case of any contamination during DNA isolation and manipulation). Those repeat families showing any significant matches to other species were eliminated from consideration.

The RepeatExplorer Clustering tool uses the 3D version of the Fruchterman and Reingold algorithm to generate graph layouts of each repeat family, in which individual reads are represented by vertices and similar reads are connected by edges (Novák et al., 2010). Therefore, clusters composed of very similar reads will form tight clusters in the graph. To quantitatively measure the similarity of reads in a repeat family, the average edge length connecting reads is output for each repeat family. A smaller average edge length denotes greater similarity among reads. To maximize the percentage of the repeat family that would be captured by a single primer and probe set for a diagnostic assay, and therefore increase the sensitivity of the assay, only repeat families forming tight clusters in the graph layout with low average edge lengths were considered for the assay. For the purposes of this paper, a 2D projection of the selected repeat family graph layout is shown (Fig. 1B).

After removing those repeat families that had similarity to sequences in other species or did not form tight clusters, the repeat family with the most reads, and therefore the most abundant in the *P. decorus* genome, was selected for primer and probe design for a quantitative PCR assay. The repeat family selected was the second most abundant in the *P. decorus* genome of the original candidates. The cluster visualization of this repeat family and its associated statistics are presented in Fig. 1. For ease of discussion, this cluster was named Pd65 and the genome, was selected for primer and probe design for a quantitative PCR assay. The repeat family selected was the second most abundant in the *P. decorus* genome of the original candidates. The cluster visualization of this repeat family and its associated statistics are presented in Fig. 1. For ease of discussion, this cluster was named Pd65 and the genome. A primer-probe set (based on repeat family Pd65 identified in Fig. 1) for amplifying and detecting *P. decorus* DNA by qPCR was designed with the PrimerQuest tool offered through Integrated DNA Technologies (Coralville, IA, USA) using standard parameters (http://www.idtdna.com/primerquest/home/index). The species-specificity of the

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Table 1

| Host ID | Host species | Infection status | Whole nematode | Fecal sample | Sputum sample | Age class | Sex | Stranding date | Source |
|---------|--------------|------------------|----------------|--------------|---------------|-----------|-----|----------------|--------|
| GFS-151 | Arctocephalus townsendi | Infected | Yes | Yes | No | Yearling | Male | 07/06/2017 | TMMC |
| CSL-13271 | Zalophus californianus | Infected | Yes | No | No | Subadult | Male | 10/30/2016 | TMMC |
| CSL-13295 | Zalophus californianus | Infected | Yes | No | No | Subadult | Male | 02/27/2017 | TMMC |
| CSL-13301 | Zalophus californianus | Infected | Yes | No | No | Adult | Female | 03/16/2017 | TMMC |
| CSL-13302 | Zalophus californianus | Infected | No | Yes | No | Adult | Male | 03/17/2017 | TMMC |
| CSL-13305 | Zalophus californianus | No known infection | No | Yes | No | Adult | Female | 03/22/2017 | TMMC |
| CSL-13308 | Zalophus californianus | Infected | Yes | No | No | Subadult | Female | 03/25/2017 | TMMC |
| CSL-13354 | Zalophus californianus | No known infection | No | Yes | No | Yearling | Female | 10/22/2017 | TMMC |
| CSL-13541 | Zalophus californianus | Infected | No | Yes | No | Adult | Female | 10/10/2017 | TMMC |
| CSL-13564 | Zalophus californianus | Infected | Yes | Yes | No | Subadult | Male | 11/06/2017 | TMMC |
| CSL-13591 | Zalophus californianus | Infected | Yes | Yes | No | Adult | Male | 12/03/2017 | TMMC |
| CSL-14040 | Zalophus californianus | Infected | No | Yes | Yes | Juvenile | Male | 10/18/2018 | TMMC |
| CSL-14066 | Zalophus californianus | Infected | No | Yes | Yes | Adult | Female | 11/04/2018 | TMMC |
| CSL-14070 | Zalophus californianus | Infected | No | Yes | Yes | Juvenile | Male | 11/05/2018 | TMMC |
| CSL-14073 | Zalophus californianus | Infected | No | Yes | Yes | Subadult | Male | 11/07/2018 | TMMC |
| CSL-14075 | Zalophus californianus | Infected | No | Yes | Yes | Yearling | Female | 11/07/2018 | TMMC |
| CSL-14083 | Zalophus californianus | Infected | No | Yes | Yes | Juvenile | Male | 11/10/2018 | TMMC |
| CSL-14084 | Zalophus californianus | Infected | No | Yes | Yes | Juvenile | Male | 11/11/2018 | TMMC |
| CSL-14089 | Zalophus californianus | Infected | No | Yes | Yes | Yearling | Female | 11/14/2018 | TMMC |
| CSL-14107 | Zalophus californianus | Infected | No | Yes | Yes | Yearling | Male | 11/28/2018 | TMMC |
| CSL-14117 | Zalophus californianus | Infected | No | Yes | Yes | Juvenile | Male | 12/05/2018 | TMMC |
| CSL-14121 | Zalophus californianus | Infected | No | Yes | Yes | Subadult | Male | 12/06/2018 | TMMC |
| CSL-14138 | Zalophus californianus | Infected | No | Yes | Yes | Subadult | Male | 12/23/2018 | TMMC |
| CSL-B | Zalophus californianus | No known infection | No | No | Yes | Adult | Female | Born in captivity | LIA |
| CSL-J | Zalophus californianus | No known infection | No | No | Yes | Adult | Male | Born in captivity | LIA |
| CSL-S | Zalophus californianus | No known infection | No | Yes | No | Adult | Female | Born in rehabilitation facility | NEAQ |
| CSL-T | Zalophus californianus | No known infection | No | Yes | No | Adult | Female | In captivity since 2013 | NEAQ |

Note: Table 1 includes data from whole nematode, fecal, and sputum samples. The infection status of the nematodes was determined by a fecal Baermann test or morphological identification at necropsy.
primer-probe set was assessed using NCBI's Primer Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The probe was labeled with a 6FAM fluorophore at the 5′ end and was double quenched using the internal quencher ZEN and the 3′ quencher 3IABkFQ (IOWA BLACK). This labeling/quenching combination has been shown to provide superior sensitivity to other probe/quencher systems (Pilotte et al., 2016).

The *P. decorus* repeat family Pd65 (Fig. 1) was amplified using 5′-GCA GAT AGG AAG AAC CCA CAA-3′ as the forward primer, 5′-AGC AGA CCT AGA CTT C-3′ as the reverse primer, and/56-FAM/AC AGC AGT C/ZEN/A TCG TGT CCA TAC CA/3IABkFQ/ as the probe. The reactions were prepared using Thermo Fisher Scientific's TaqMan® Universal PCR Master Mix (Waltham, MA, USA). All samples were run in quadruplicate. Mean Cq (quantification cycle) and standard deviation reported in the results were calculated using all four data points from each sample to reduce error.

2.7. Assay specificity and sensitivity testing

To test the species-specificity of the Pd65 primer-probe set, DNA from a variety of nematode species, including both metastrongylid and non-metastrongylid parasites, was qPCR amplified using the same conditions described above. DNA isolated from a confirmed *P. decorus* nematode from marine mammal host CSL-13308 was used as the positive control (Table 1). Whole nematodes used as negative controls were obtained from the following sources: Baylor College of Medicine (Anclyostoma duodenale and Necator americanus), Filariasis Research Reagent Resource Center (Brugia malayi), The Marine Mammal Center (Ostertagia circumlitis), Mystic Aquarium (Pseudoterranova decipiens), and National Marine Life Center (Acanthocheilonema spirocauda and Contracaecum osculatum). To determine the limits of detection for the assay, reactions were run with total input DNA of: 1 ng, 0.1 ng, 0.01 ng, 0.001 ng (1 pg), 0.0001 ng, 0.00001 ng, and 0.000001 ng (1 fg). Detection was reliable at 0.001 ng (1 pg) and had fairly low standard deviations; thus, this concentration was used for both specificity testing and testing of whole worm DNA isolates.

2.8. Testing of fecal and sputum samples

Fecal and sputum samples from otariids with known *P. decorus* infections and without known infections were provided by The Marine Mammal Center (Table 1). Fecal samples from one of the primary host species, Zalophus californianus, from the New England Aquarium were used as negative fecal controls because these animals have been in captivity for many years on a diet free of nematode-infected fish and were known to be free of *P. decorus* infections (Table 1). Similarly, sputum samples from Zalophus californianus were provided by the Long Island Aquarium for use as a negative control, as these sea lions had been born in captivity, fed on uninfected fish throughout their life, and were known to be free of *P. decorus* infections (Table 1). DNA from fecal samples and sputum samples was extracted as previously described. Pd65 qPCR was performed using 1 ng of template DNA per reaction and the reaction conditions described above.

3. Results

3.1. Specificity

To test the Pd65 qPCR assay for specificity, an array of nematode species ranging in phylogenetic distance from *Parafilaroides decorus* were tested, including Ostertagia circumlitis within the same superfamily (Metastrongyloidae), Necator americanus within the same order (Strongyldia), Acanthocheilonema spirocauda and Brugia malayi in the same class (Secernentea), and several nematode species outside of the Secernentea class (Anclyostoma duodenale, Contracaecum osculatum, and Pseudoterranova decipiens). Critically, we tested species that infect the same hosts as *P. decorus* (such as Contracaecum osculatum) to ensure that the assay does not identify false positives from species that will be present in the material being tested from the host species. Amplification was detected only in the *P. decorus* positive control, suggesting that the assay has high specificity at least to the genus level (Table 2). Samples from other *Parafilaroides* species that were not *P. decorus* were not available, thus specificity testing within the genus was not possible.
3.3. Fecal samples from necropsied animals

Cq values obtained from the Pd65 qPCR on genomic DNA isolated from fecal samples containing unknown quantities of *P. decorus* DNA were used to estimate the amount of *P. decorus* DNA that was in the sample. The amount of *P. decorus* DNA present in 1 ng of total fecal DNA (which would also include host DNA, bacterial DNA, etc.) is estimated in Table 4.

3.4. Paired fecal and sputum samples

Cq values obtained from Pd65 qPCR on genomic DNA isolated from paired fecal and sputum samples containing unknown quantities of *P. decorus* DNA were used to estimate the amount of *P. decorus* DNA that was in each sample. The amount of *P. decorus* DNA present in the 1 ng of total fecal and sputum DNA used in the Pd65 qPCR assay is estimated with a 95% confidence interval of ± 0.39 ng in Table 5.

*P. decorus* detected in both sample types and there was never detection in negative fecal controls or negative sputum controls. Neither sample type consistently produces higher DNA concentration estimates than the other.

4. Discussion

4.1. Specificity

The qPCR assay developed here appears to be genus specific to *Parafilaroides*, as no other nematode species were detected by the assay. We cannot say definitively that the assay is species-specific to *P. decorus* because no nematode species of the same genus (*Parafilaroides*) were tested. Due to the extremely small size of the parasite and their tendency to be embedded in the pulmonary tissue, most facilities do not collect *Parafilaroides* specimens during necropsy. However, no other *Parafilaroides* species are known to infect the pinnipeds classically infected (the California sea lion, Guadalupe fur seal, and Northern fur seal) (Kuzmina et al., 2018). Whether our assay would detect these other species is unknown. While *Parafilaroides* is not routinely collected during most necropsies, we hope to obtain *Parafilaroides* samples of other species in the future to determine if the assay is species-specific, or if it amplifies DNA from other *Parafilaroides* species as well. Despite these unknowns, the level of specificity of the Pd65 assay still surpasses the barcoding PCR tests currently in use (Rishniw et al., 2006).

4.2. Sensitivity

In pure *P. decorus* genomic DNA samples, detection of as little as 10 fg, or 0.00001 ng, was possible (Table 3). To discern the relationship between DNA concentration and number of larvae, an exact larval count is needed in biological samples. While such data do not exist for *P. decorus*, a previous *Ascaris lumbricoides* study have found roughly 1000 eggs yielded 1 ng of nematode DNA from fecal samples (Easton et al., 2017). This would suggest that our qPCR assay can detect the presence of 0.01 eggs, assuming *A. lumbricoides* and *P. decorus* have similar quantities of DNA per egg or larva respectively, and assuming
efficient isolation of DNA from biological material.

4.3. Detection of *P. decorus* in fecal samples

Using the Pd65 qPCR assay, *P. decorus* was detected in fecal samples from all animals with known *P. decorus* infections. Furthermore, all of the estimates of the amount of *P. decorus* DNA found in the fecal samples were less than the total 1 ng of DNA in the fecal sample (at a maximum of 48% of the total DNA), which suggests that the *P. decorus* DNA was detected in the fecal samples but not in the sputum samples.

### Table 4

| *P. decorus* infection status | Mean Cq | Cq standard deviation | Estimated input *P. decorus* DNA (ng) | Host ID  |
|-----------------------------|---------|-----------------------|--------------------------------------|---------|
| Infected                    | 18.17   | 1.2                   | 0.27                                 | GFS-151 |
| 25.41                       | 0.024   | 0.0018                | CSL-13902                            |
| 22.93                       | 0.065   | 0.01                  | CSL-13541                            |
| 17.32                       | 0.048   | 0.48                  | CSL-13564                            |
| 25.17                       | 0.036   | 0.0021                | CSL-13591                            |
| 36.33                       | 0.91    | 9.40E-07              | CSL-14040                            |
| 24.60                       | 0.12    | 0.0031                | CSL-14066                            |
| 24.88                       | 0.57    | 0.0026                | CSL-14070                            |
| 18.13                       | 0.14    | 0.27                  | CSL-14073                            |
| 23.86                       | 0.28    | 0.0052                | CSL-14075                            |
| 33.80                       | 0.093   | 5.40E-06              | CSL-14083                            |
| 32.82                       | 2.9     | 1.16E-05              | CSL-14084                            |
| 17.15                       | 0.35    | 0.54                  | CSL-14089                            |
| 19.37                       | 0.57    | 0.12                  | CSL-14107                            |
| 28.71                       | 0.17    | 0.00018               | CSL-14117                            |
| 25.31                       | 0.64    | 0.0019                | CSL-14121                            |
| 20.99                       | 0.60    | 0.071                 | CSL-14138                            |
| No known infection          | No detection | -                 | CSL-13305                            |
| 25.47                       | 0.084   | 0.0017                | CSL-13534                            |
| No known infection*         | No detection | -                 | CSL-S                                |
|                            | No detection | -                 | CSL-T                                |

### Table 5

| Host ID  | Feces mean Cq | Sputum mean Cq | Feces estimated input target DNA (ng) | Sputum estimated input target DNA (ng) |
|----------|---------------|----------------|--------------------------------------|----------------------------------------|
| CSL-14040| 36.33         | 26.08          | 9.45E-07                             | 0.001132                               |
| CSL-14066| 24.6           | 16.55          | 0.00314                              | 0.8204                                 |
| CSL-14070| 24.88         | 31.83          | 0.00258                              | 2.12E-05                               |
| CSL-14073| 18.13         | 20.63          | 0.2741                               | 0.04866                                |
| CSL-14075| 23.86         | 28.11          | 0.005244                             | 0.0002778                              |
| CSL-14083| 33.8           | 31.03          | 5.42E-06                             | 3.68E-05                               |
| CSL-14084| 32.82         | 24.65          | 1.07E-05                             | 0.003039                               |
| CSL-14089| 17.15         | 29.69          | 0.5406                               | 9.33E-05                               |
| CSL-14107| 19.37         | 18.7           | 0.1166                               | 0.1858                                 |
| CSL-14117| 28.71         | 28.26          | 0.0001832                            | 0.0002497                              |
| CSL-14121| 25.31         | 32.26          | 0.001925                             | 1.58E-05                               |
| CSL-14138| 20.09         | 26.03          | 0.07093                              | 0.001117                               |
DNA estimates are likely correct, since most of the DNA in a fecal sample should be host and bacterial DNA. Unfortunately, we cannot directly estimate the concentration of larvae in the feces because the correlation between egg DNA concentration in the feces and number of larvae is currently unknown for *P. decorus*.

Host CSL-13534, notably, was not known to have a *P. decorus* infection based on the fecal testing and necropsy data (Tables 1 and 4). Infections with these particularly small nematodes can be easily missed in necropsies and, more importantly, in fecal Baermann tests that can be processed while the animal is still alive. This demonstrates that the Pd65 assay can detect infections that would otherwise be missed. This scenario is similar to soil-transmitted helminth molecular diagnostic assays, which are often able to detect infections that are missed by visual fecal examination methods due to storage methods causing eggs to break down or human error (Pilott et al., 2016, 2019; Easton et al., 2017).

### 4.4. Detection of *P. decorus* in paired fecal and sputum samples

*P. decorus* detection was achieved by the Pd65 qPCR assay in both fecal and sputum samples from all hosts tested with known *P. decorus* infections. Furthermore, both feces and sputum samples were equally sensitive for detection of *P. decorus* DNA (Table 5). This suggests that either or both of these sample types could be used to diagnose *P. decorus* infection using this assay. However, feces and sputum were not necessarily collected on the same day, and therefore may have been collected during different points in the course of the infection, or even during different points in treatment of the infection. Thus, the true relationship between Pd65 qPCR detection in feces and sputum as indicators of *P. decorus* infection warrants further investigation. A follow-up study to examine this relationship using paired fecal and sputum samples collected at the same point in time is necessary.

### 5. Conclusions

The Pd65 repeat-based qPCR assay will enable the marine mammal rehabilitation community to use fecal or sputum samples to diagnose and treat live animals with confidence in the identity of *P. decorus* infections and with far greater specificity and sensitivity than is possible with fecal Baermann tests or other microscopy-based methods. Even with the limited number of samples, the Pd65 qPCR assay has already uncovered an undetected *P. decorus* infection in a sea lion that had previously gone undiagnosed.

The Pd65 qPCR assay also has the potential for use in screening wild populations for infection using scat to obtain data on the epidemiology of infection under natural conditions. As demonstrated here, the assay is able to identify a *P. decorus* infection and the comparative level of burden in live animals using either feces or sputum. This information can be used in the future to best determine the optimal treatment to aid in the rehabilitation and release of infected animals.

### Declaration of competing interest

None.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.04.012.

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