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A novel quantitative real-time PCR diagnostic assay for seal heartworm (*Acanthocheilonema spirocauda*) provides evidence for possible infection in the grey seal (*Halichoerus grypus*)

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ABSTRACT

The distinct evolutionary pressures faced by Pinnipeds have likely resulted in strong coevolutionary ties to their parasites (Leidenberger et al., 2007). This study focuses on the phocid seal filarial heartworm species *Acanthocheilonema spirocauda*. *A. spirocauda* is known to infect a variety of phocid seals, but does not appear to be restricted to a single host species (Measures et al., 1997; Leidenberger et al., 2007; Lehnert et al., 2015). However, to date, seal heartworm has never been reported in grey seals (*Halichoerus grypus*) (Measures et al., 1997; Leidenberger et al., 2007; Lehnert et al., 2015). The proposed vector for seal heartworm is *Echinophthirius horridus*, the seal louse. Seal lice are known to parasitize a wide array of phocid seal species, including the grey seal. With the advent of climate change, disease burden is expected to increase across terrestrial and marine mammals (Harvell et al., 2002). Accordingly, increased prevalence of seal heartworm has recently been reported in harbor seals (*Phoca vitulina*) (Lehnert et al., 2015). Thus, the need for improved, rapid, and cost-effective diagnostics is urgent. Here we present the first *A. spirocauda*-specific rapid diagnostic test (a quantitative real-time PCR assay), based on a highly repetitive genomic DNA repeat identified using whole genome sequencing and subsequent bioinformatic analysis. The presence of an insect vector provides the opportunity to develop a multifunctional diagnostic tool that can be used not only to detect the parasite directly from blood or tissue specimens, but also as a molecular xenomonitoring (XM) tool that can be used to assess the epidemiological profile of the parasite by screening the arthropod vector. Using this assay, we provide evidence for the first reported case of seal heartworm in a grey seal.

1. Introduction

Parasites infect almost all life forms on the planet and marine mammals are no exception to this rule. Marine mammal parasites vary from helminthes, to arthropods, to protozoans (Dailey, 2005). Marine mammals are diverse organisms with unique character traits forged during their ancestors' transition from land to sea. Marine mammals are comprised of three major orders: Cetartiodactyla (cetaceans), Sirenia (dugongs, manatees) and Carnivora (pinnipeds). Unlike the Certartiodactyla and Sirenia, Pinnipeds have adopted a more amphibious lifestyle. This unique adaptation not only imposes evolutionary pressure on pinnipeds, but also on the parasitic fauna they host (Leidenberger et al., 2007). These parasites often share strong co-evolutionary ties to their hosts, having adapted with them as the marine mammals transitioned from land back to the sea millions of years ago (Leidenberger et al., 2007; Lehnert et al., 2010).

Heartworms are a serious health concern in marine mammals (Dailey, 2005). Heartworms like *Dirofilaria immitis* are known to infect a wide range of hosts including most canids and a variety of carnivores including sea lions and harbor seals (Hubert et al., 1980; Gortazar et al., 1994; Furtado et al., 2010; Dantas-Torres and Otranto, 2013). The focus of this work is, however, the seal heartworm (*Acanthocheilonema spirocauda*). Seal heartworm is a filarial parasite that infects phocid seals, including the harbor seal (*Phoca vitulina*). While the parasite has not yet emerged as a significant threat to seal populations, infection with seal heartworm can result in similar pathology to infections with *D. immitis,* including anorexia, fatigue, heart and lung complications, and potentially death (Taylor et al., 1961; Leidenberger et al., 2007). Seal heartworm is believed to have coevolved with its phocid host some 45 million years ago (Leidenberger et al., 2007) and is believed to be

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transmitted by the seal louse (*Echinophthirius horridus*) (Geraci and Fortin, 1981; Leidenberger et al., 2007). Relatively little epidemiological data for the parasite exists, as research has been limited. Few studies have focused on prevalence of infection, and available data has high variation ranging from 65% reported in harbor seals on the coast of the Netherlands (1963), to 25% in the Baltic Sea (1991), and 11.4%, in the North Sea (1992) (van den Broek, 1963; Claussen et al., 1991; Lunneryd, 1992; Lehnert et al., 2015). A more recent study of harbor seals from the North and Baltic Seas found temporal variations in prevalence, with infection levels ranging from 11% to 57% depending on the month and year of sampling (Lehnert et al., 2015). Thus, the true burden of the disease remains undetermined. To better understand the prevalence of the disease, sensitive diagnostic tools are needed. While it has been proposed that the prevalence of seal heartworm is increasing and possibly seasonal, baseline data is conflicting from the aforementioned studies (Lehnert et al., 2015). In the face of climate change and the resulting inevitable spread of infectious diseases, novel diagnostic tools will be essential in monitoring heartworm (Harvell et al., 2002).

Proper treatment, management, and control of infectious disease relies on sensitive, reliable, and rapid diagnostics with exquisite species-specificity to guide proper clinical management (Banoo et al., 2008; Caliendo et al., 2013). In the case of seal heartworm, good diagnostics will allow for better care of live animals in rehabilitation or less invasive methods of monitoring prevalence. The main goal of this research project was not to generate a definitive method of identifying a broad array of parasites, but rather to quickly, reliably, and accurately diagnose seal heartworm (*A. spirocauda*). As molecular approaches are becoming more popular diagnostic tools, the focus of this work was to generate a novel DNA based diagnostic (Powers, 2004; Edvinsson et al., 2006; Banoo et al., 2008; Mejia et al., 2013; Papaiakovou, 2014; Alhassan et al., 2015; Ricciardi and Ndao, 2015; Pilotte et al., 2016a). Traditional PCR-based diagnostics have relied primarily on moderately repeated sequences such as mitochondrial genes or ribosomal RNA-encoding genes (rDNA) which have limited resolution at the species level (Gatehouse and Malone, 1998; Mejia et al., 2013; Liu et al., 2014; Alhassan et al., 2015; Ricciardi and Ndao, 2015; Pilotte et al., 2016a).

A DNA barcode is a simple genetic signature for a species and tends to be comprised of nuclear and/or mitochondrial gene sequences with sufficient but not excessive variation (Floyd et al., 2002; Blaxter, 2004; Moritz and Cicero, 2004; Powers, 2004; Waugh, 2007; Borisenko et al., 2009; Packer et al., 2009; Casiraghi et al., 2010). Two widely used DNA barcodes are the small (18S) ribosomal subunit (SSU) and the nuclear internally transcribed spacer region 2 (ITS2) (Harris and Crandall, 2000; Blouin, 2002; Floyd et al., 2002; Álvarez, 2003; Young and Coleman, 2004; Schultz, 2005; Holterman, 2006; Pace, 2009; Nassonova et al., 2010; Agüero-Chapin et al., 2011; Hill et al., 2014). While SSU is often viewed as a “gold standard” for phylogenetics (Floyd et al., 2002; Holterman, 2006; Pace, 2009), ITS2 is widely regarded as an excellent barcoding sequence, however problems in resolution exist for these single gene barcodes (Harris and Crandall, 2000; Álvarez, 2003; Coleman, 2003; Young and Coleman, 2004; Nassonova et al., 2010; Agüero-Chapin et al., 2011). The utility of these two sequences in identifying seal nematode parasites are assessed in this study.

Other sequences exist in the genome at much higher copy numbers and thus may give better sensitivity and specificity for a molecular diagnostic. Nematode genomes are known to contain high levels of repetitive sequences, often containing non-coding repeats that can be have up to 1000 or more copies per haploid genome (The C. elegans Sequencing Consortium, 1998; Thanchomnang et al., 2008; Pilotte et al., 2016a). These repeats are often non-coding and are therefore less subject to evolutionary conservation, resulting in divergence between even closely related species (Pilotte et al., 2016a). Whereas ITS2 generally has no more than a few hundred copies per haploid genome, these non-coding repetitive sequences (NCR) can exist in thousands of copies per haploid genome (Gatehouse and Malone, 1998; Pilotte et al., 2016a). Thus, the NCRs lend both improved specificity and sensitivity to DNA-based diagnostic assays.

To design a diagnostic assay based on these NCRs, the full genome of seal heartworm was sequenced using massively parallel sequencing. Another advantage of using NCRs for diagnostic design is the requirement for relatively low coverage genome sequencing. Prior to the development of new genome sequencing methods and bioinformatic search tools, identification of repeats was often computationally expensive and laborious. (McReynolds et al., 1986; Zhong et al., 1996; Vargas et al., 2000; Pilotte et al., 2016a). However, with the rise of massively parallel sequencing and the burgeoning development of new analytic software and bioinformatic tools, repeat identification has become significantly easier and can be performed on non-annotated, unassembled genomic data (Novák et al., 2010, 2013; Treangen and Salzberg, 2011; Subirana and Messeguer, 2013; Pilotte et al., 2016a). Several recent tools have been developed for repeat identification, each with their own specific purpose (Roset et al., 2003; Mayer, 2006; Mayer et al., 2010; Novák et al., 2017, 2013). RepeatExplorer is a freely available bioinformatic pipeline that identifies highly repetitive sequences from raw FASTQ data using a graph-based clustering algorithm (Novák et al., 2010, 2013). Once a sequence is identified and selected, a TaqMan-based quantitative real-time PCR assay can be designed to detect that sequence. Using this methodology, we have developed and successfully implemented a real-time qPCR assay to detect parasite DNA in whole nematode isolates, seal lice, and infected blood samples. This represents the development of the first rapid molecular diagnostic assay for seal heartworm. Using this assay on field-collected samples, we present evidence of possible seal heartworm infection in the grey seal (*Halichoerus grypus*).

2. Materials and methods

2.1. Parasite material and DNA isolation

A. spirocauda and other nematode parasites (n = 17), lice (n = 25), and blood samples (n = 1) were obtained from stranded, deceased seals collected by the New England Aquarium (NEAQ, Quincy MA), Northeast Fisheries Science Center (NEFSC, Woods Hole MA), and the National Marine Life Center (NMLC, Bourne MA) (Supplementary Table 1). For each host, a single nematode specimen was collected and preserved in ethanol by the respective agencies. All host seals were stranded/by-caught off the Northeast coast (Massachusetts, New Hampshire) of the Atlantic Ocean, with the exception of P-Pr-11-007 which was collected in California. Each identification number represents the single host animal from which the nematode specimen was collected (Supplementary Table 1). When possible, nematodes were morphologically identified by the National Marine Life Center (Dr. Rogers Williams). These samples were transferred to Smith College with the permission of 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 allows transfer of marine mammal parts for scientific research purposes.

Total genomic DNA from *A. spirocauda* was isolated following established protocols, using organic extraction and ethanol precipitation (Keroack et al., 2016). DNA was quantified using the high sensitivity Qubit fluorometric quantitation assay (Thermo Fisher Scientific, Waltham MA). DNA quality for PCR was validated using SSU and ITS2 primers specific to nematodes. SSU and ITS2 PCR products were sequenced to confirm morphological species identifications (Supplementary Table 1). The 18S small subunit rRNA (SSU) was amplified using previously reported primers (Floyd et al., 2002). Amplification was done using 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, 52 °C annealing for 30 s, and 72 °C extension for 1.5 min, followed by a final extension for 10 min at 72 °C. ITS2 was amplified using previously published primers (Rishniw et al., 2006). Amplification was done using 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.

Louse DNA was isolated following the same protocol as for parasite genomic DNA with an additional preliminary homogenization step prior to incubation with proteinase K. DNA from mock infected blood samples was isolated using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocols. Quality was assessed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, V 3.8, Waltham, MA).

2.2. Genome sequencing (Illumina)

A. spirocauda genomic DNA was pulse-sonicated using a cup-horn sonicator (QSonica, Newton, CT) for 2.5 min to generate fragmented DNA. Fragmentation was confirmed using 1% agarose gel electrophoresis. The resulting fragment sizes ranged between 200 and 1500 base pairs. Fragmented total genomic DNA was then prepared for sequencing using the NEBNext Ultra® II kit (New England BioLabs, Ipswich, MA) following the manufacturer's instructions. Total input DNA for library construction was 28 ng. Library quality was validated using the Agilent Bioanlyzer High Sensitivity DNA kit (#5076-4626, Agilent, Santa Clara, CA) and was sequenced using MiSeq v2 500 cycle reagents (MS-102-2003, Illumina, San Diego, CA**)** and a 1% PhiX control.

2.3. Repeat DNA analysis

A. spirocauda repeat DNA selection was done following an established, previously published workflow using standard parameters for all tools (Novák et al., 2010, 2013; Pilotte et al., 2016a). Raw FASTQ files were uploaded to the Galaxy RepeatExplorer server (http://www.repeatexplorer.org/) (Novák et al., 2013). Files were then converted to the FASTQsanger format using the FASTQ Groomer tool. Paired reads were then combined using the FASTQ Interlacer tool and adapter sequences were removed using CutAdapt. Finally, the file was converted from FASTQ to FASTA format and sampled to 400,000 sequences (Blankenberg et al., 2010). Repetitive sequences were identified using the RepeatExplorer Clustering tool. Clusters that showed a tight starburst shape were selected as ideal clusters; spherical clusters represent those of shorter sequence length, ideal for qPCR (Fig. 1). Repetitive sequences were also analyzed using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure that the repeat sequences selected were not ribosomal, mitochondrial, or a close match to other related parasite species or to marine mammal host DNA.

2.4. Quantitative PCR assay design

A primer-probe set (based on cluster 20, Fig. 1) for amplifying and detecting *A. spirocauda* DNA was designed using the PrimerQuest tool offered through IDT (http://www.idtdna.com/primerquest/home/index) using standard parameters. The species-specificity of the 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) (Integrated DNA Technologies, Coralville, IA). This labeling/quenching combination has been shown to provide superior sensitivity to other labeling systems (Pilotte et al., 2016a).

The *A. spirocauda* repeat selected (cluster 20, Fig. 1) was amplified using 5′- AGGAACTGCATGGAGTGAAG-3′ as the forward primer, 5′-CTCCTTCAATTTCCTCTCCTTCT -3′ as the reverse primer, and/56-FAM/AGGAACTGA/ZEN/AGGAACCGAAGAAACTGA/3IABkFQ/ as the probe. The reactions were prepared using the TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) with 200 nM forward and reverse primer concentrations, and 125 nM probe concentration, following the manufacturer's protocols. Cycling conditions were as prescribed by the manufacturer with an anneal and extend temperature of 66 °C. One nanogram of input DNA was used for each reaction. Total reaction volume for all reactions was 20 µL. All reactions were run on a StepOne Plus Real-Time PCR system (LifeTechnologies, Carlsbad, CA). All subsequent Real-Time PCRs were run following these parameters.

2.5. Specificity and sensitivity testing

Reactions were run using DNA from a variety of nematode species, including both filarial and non-filarial parasites to test the species-specificity of the primer-probe set. DNA isolated from a confirmed *A. spirocauda* nematode (P-Pr-13-104) was used as a positive control. For each reaction, 1 ng of parasite DNA was used as the template DNA. Assays were tested in duplicate using 1 ng of parasite genomic DNA (gDNA) as template. 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, 0.0001 ng, 0.00001 ng, and 0.000001 ng. Reagents and cycling conditions were used as described above.

2.6. Testing of whole worm isolates

Whole worms (a single nematode per seal host) were collected from

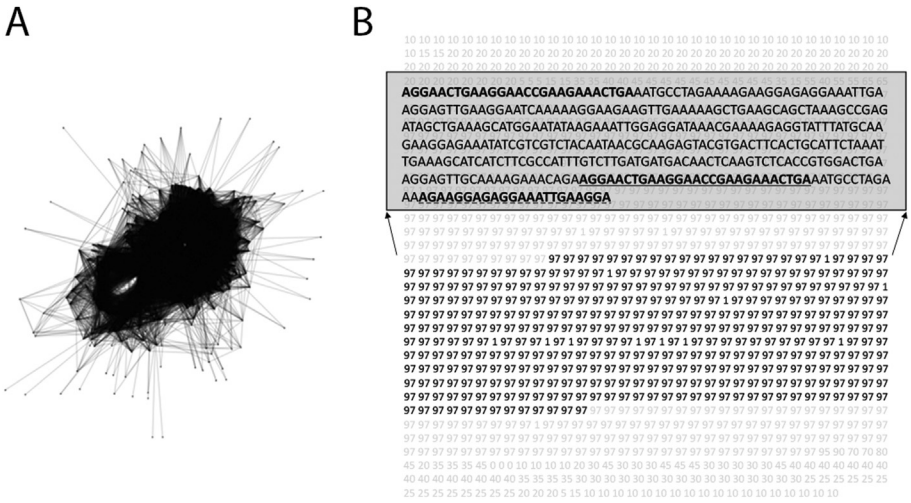

Fig. 1. A: graphical representation of cluster 20 (C20). B: representation of the selected contig and the sequence used to design the C20 quantitative real-time PCR assay. The forward primer is in bold, the reverse primer is indicated by a dotted underline, and the double-quenched probe is underlined.

Table 1
C20 qPCR assay specificity and sensitivity.

a. Specificity testing

Species	DNA (ng)	Mean Ct Value	SD
A. spirocauda (P-Pr-13-104)	1	16.75	0.01
A. viteae	1	nd[a]	nd
D. immitis	1	nd	nd
H. delphini (P-Pr-12-050)	1	nd	nd
A. simplex (DO 8135)	1	nd	nd
B. pahangi	1	nd	nd
D. odendhali (P-Pr-13-007)	1	nd	nd
O. circumlitus (P-Pr-13-024)	1	nd	nd
C. boopis (P-Pr-11-018)	1	nd	nd
No template control (NTC)	–	nd	nd

b. Sensitivity testing

Species	DNA (ng)	Mean Ct Value	SD
A. spirocauda (P-Pr-13-104)	1	15.98	0.34
A. spirocauda (P-Pr-13-104)	0.1	23.92	0.07
A. spirocauda (P-Pr-13-104)	0.01	28.55	0.25
A. spirocauda (P-Pr-13-104)	0.001	35.02	1.37
A. spirocauda (P-Pr-13-104)	0.0001	38.58	0.02
A. spirocauda (P-Pr-13-104)	0.00001	nd	nd
A. spirocauda (P-Pr-13-104)	0.000001	nd	nd
No Template Control (NTC)	–	nd	nd

[a] nd = not detected.

deceased seals from either the heart (n = 9) or the lungs (n = 8) as previously described (Supplementary Table 1). Nematodes were collected during necropsy by staff at either NMLC, NEAQ, or NEFSC and preserved in ethanol and frozen. These samples were then processed at Smith College for DNA. Following DNA isolation, 1 ng of DNA was used for each qPCR reaction. Specimens were collected from multiple seal species over a broad geographic range (Supplementary Table 1). Whole nematodes used as negative controls were obtained from the following sources: *Acanthocheilonema viteae* (BEI resources, Filariasis Research Reagent Resource Center (FR3)), *Dirofilaria immitis* (BEI, FR3), *Brugia pahangi* (BEI, FR3), *Anisakis simplex* (NEFSC), *Dipetalonema odendhali* (NMLC), *Halocercus delphini* (NMLC), *Crassicauda boopis* (NMLC).

2.7. Testing of louse specimens

Morphologically identified lice were provided by the National Maine Life Center and the New England Aquarium (Table 2). Lice were homogenized using a 1 mL Ten-Broeck homogenizer (Thermo Fisher Scientific, Waltham, MA), and DNA was extracted as previously described. Real-time PCR was performed using 1 ng of template DNA per reaction.

2.8. Testing detection of A. spirocauda DNA added to P. groenlandicus blood

To test the applicability of our assay in screening seal blood, we generated mock "infected" blood samples. Harp seal (*Pagophilus groenlandicus,* ID: NEAQ 16-013) blood from an uninfected juvenile seal stranded in Maine (died overnight after transport to Quincy, MA for rehabilitation) was provided by the New England Aquarium following previously described NOAA guidelines. Mock infected blood samples were generated by addition of purified *A. spirocauda* DNA to whole seal blood at final concentrations of 0.05 ng/μL, 0.005 ng/μL, and 0.0005 ng/μL. Total DNA was extracted from spiked blood samples using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following manufacturer's protocols. DNA quality was assessed using a Nanodrop 1000 spectrophotometer V3.8 (Thermo Fisher Scientific, Waltham, MA), and quantified using a Qubit Fluorometer 2.0 (Thermo Fisher Scientific, Waltham, MA), using a dsDNA BR assay kit (Invitrogen, Carlsbad, CA). Real-time PCR reactions were run using 1 ng of template DNA. In addition to the mock infected samples, a standard curve of *A. spirocauda* DNA was run to quantify the amount of parasite DNA in the mock blood samples. Serial dilutions of *A. spirocauda* DNA were included on the reaction plate this purpose: 1 ng, 0.1 ng, 0.01 ng, 0.001 ng, 0.0001 ng, and 0.00001 ng. Estimates of the amount of DNA in spiked blood samples were calculated using Prism 7 software (GraphPad Software, La Jolla, CA) with the "interpolate unknowns from a standard curve" function with standard parameters.

Table 2
C20 qPCR results for 20 whole worm isolates.

Nematode specimen (host sp.)	Ct	SD
P-Pr-13-108 (*P. vitulina*) (positive control)	16.66	0.13
P-Pr-13-106 (*H. grypus*)	24.17	0.45
P-Pr-13-104 (*P. vitulina*)	22.64	0.25
P-Pr-11-007 (*P. vitulina*)	22.85	0.66
DO 5476 (*P. vitulina*)	24.83	1.49
NEAQ-14-048 H (*P. vitulina*)	18.94	0.80
NEAQ-14-048 L (*P. vitulina*)	nd[a]	nd
NEAQ-14-030 RL (*H. grypus*)	nd	nd
MH-07-513 (*P. vitulina*)	30.84	1.02
MH-00-797 (*C. cristata*)	nd	nd
NEAQ-13-102 (*H. grypus*)	nd	nd
NEAQ-14-030 LL (*H. grypus*)	nd	nd
MH-02-568 (*C. cristata*)	nd	nd
NEAQ-12-180 (*P. vitulina*)	nd	nd
MH-05-440 (*P. groenalandicus*)	nd	nd
MH-06-588 (*C. cristata*)	nd	nd
NEAQ-13-151 (*P. vitulina*)	20.40	0.34
D. immitis (negative control)	nd	nd
A. viteae (negative control)	nd	nd
C. boopis (negative control)	nd	nd

[a] nd = not detected.

3. Results

3.1. Barcoding

Molecular barcodes consisted of a combination of the internally transcribed spacer region 2 (ITS2) and the small ribosomal subunit (SSU). Barcoding was done on the 17 *A. spirocauda* candidate nematodes tested in this study as a means of comparing standard barcodes to the results of our new *A. spirocauda* real-time PCR test (Supplementary Table 1). Seven of the 17 nematodes tested were positively identified as *A. spirocauda* based on essentially perfect matches to *A. spirocauda* ITS2 sequences in GenBank. These same seven also had outstanding SSU matches to *Acanthocheilonema viteae* (prior to this study, there were no SSU sequences in GenBank for *A. spirocauda*, so *A. viteae* is the closest match). (P-Pr-13-108, P-Pr-13-106, P-Pr-11-007, MH-07-513, DO 5476, P-Pr-13-104). Of the remaining 10 nematodes, three are clearly *Otostrongylus circumlitus* (NEAQ-14-030 Hg RL, NEAQ-14-030 Hg LL, NEAQ-12-180-Pv), two are likely *Halocercus invaginatus* (NEAQ-14-048 L, MH-02-568), and five gave inconsistent results between the two barcodes (NEAQ-14-048 H, NEAQ-13-102 Hg, MH-05-440-Pg, MH-06-588-Cc, MH-00-797-Cc) (Supplementary Table 1).

3.2. Genome sequencing

The genome of *A. spirocauda* was sequenced in order to identify species-specific high copy number repeat DNA clusters. *A. spirocauda* DNA was sequenced on the Illumina® MiSeq platform using paired ends (2 × 250), and gave 22,746,574 sequence reads, with 20,518,418 passing filter (1st: 90.30% ≥ Q30; 2nd: 85.58 ≥ Q30; Avg: 87.94% ≥ Q30). For both reads, the cluster passing filter percentage was ideal at 90.21%. The cluster density on the flow cell was at the

maximum recommended level, 1210 L/MM^2. Raw sequence data was quality assessed using Basespace FASTQC (basespace.illumina.com). Genome assembly is not required for analysis in the RepeatExplorer pipeline and was not done. Raw FASTQ files were deposited in the Sequence Read Archive (SRA: study, SRP125495/sample, SRS2709986).

3.3. RepeatExplorer generates species-specific repeat DNA clusters

Clustering analysis using a 1,000,000 read subset of the genomic reads yielded 60 repeat DNA clusters. Cluster 20 (C20) was selected for the primer-probe design for the real-time PCR assay since more sequence reads mapped to this cluster than any other (Fig. 1). The graphical output of C20 is also shown in Fig. 1. A cluster represents a graph of the sequence reads that belong to a repetitive DNA element. Generation of the cluster occurs by pairwise comparison of every read in the data set to identify similarities. On the right is numerical output representing C20. Higher numbers represent more abundant nucleotides, and these are used for qPCR assay design (Novák et al., 2010; Pilotte et al., 2016a). BLASTN analysis of C20 yielded no similarity to any nematode parasite, and the cluster sequence can be considered species-specific. Additionally, BLASTN analysis against all nematode sequences available in GenBank yielded no results of significant similarity. Further, the region within C20 used for assay design returned no results of significant similarity to any marine mammal host DNA. The primers and probe were designed using this C20 sequence as shown in Fig. 1.

3.4. C20 qPCR assay specificity and sensitivity

In order to ensure species-specificity of the C20 assay, several species of nematodes were tested. The species selected for testing represented a wide range of nematode species, including both filarial and non-filarial species. The selected species included close relatives of *A. spirocauda* (such as *A. viteae* and *D. odendhali*), other commonly seen marine mammal nematode parasites (the lung worm *O. circumlitus* and the gastrointestinal worm *Anisakis simplex*), and other known heartworm species seen in seals (*D. immitis*). In all cases, amplification was only detected in reactions containing *A. spirocauda* DNA (Table 1a). These results demonstrate exquisite species-specificity that surpasses currently available barcoding PCR assays (Casiraghi et al., 2001; Rishniw et al., 2006). Currently, the only molecular diagnostic tool for seal heartworm is a COI PCR (Lehnert et al., 2015). Previous studies have demonstrated that COI is often insufficient in resolving species identity when used alone (Casiraghi et al., 2001, 2004).

To determine the detection limits of the assay, DNA concentrations ranging from 1 ng to 1 fg were tested. While amplification was detected at the lower limit (both 1 and 10 fg), Ct values showed high deviation between replicates at these low levels. Thus, the lowest concentration that can be confidently detected, was 100 fg (0.0001 ng). Consistent detection was observed at all other concentrations above 0.0001 ng (Table 1b).

3.5. C20 qPCR testing of nematode specimens

Field specimens were screened with the C20 quantitative real-time PCR assay to demonstrate the ability of the test to identify DNA isolated from individual parasites. In total, 20 individual worms collected from either seal hearts or lungs were tested from a range of host seal species (Table 2). Of these, 8 nematodes were positive by the C20 diagnostic assay. Of these 8 positives, 7 were identified as *A. spirocauda* by ITS2 and similar to *A. viteae* and other filarial nematodes by SSU. The eighth nematode was collected from the heart of harbor seal NEAQ-14-048 H and was not identified as *A. spirocauda* by ITS2, but was similar to *A. viteae* and other filarial nematodes by SSU. This parasite was in poor condition and was engulfed in host tissue. For longer targets like the barcodes, low quality (short) DNA can severely impact PCR and sequencing results. However, the short target amplified by our C20 assay facilitates identification using even poor quality DNA as a template. Of the eight *A. spirocauda* C20 positives, three had previously been morphologically confirmed to be *A. spirocauda* by Sea Rogers Williams of the National Marine Life Center: P-Pr-13-108, P-Pr-11-007, P-Pr-13-104. Nematodes from NEAQ-14-048H, MH-07-513, MH-06-588, and P-Pr-13-106 were not examined morphologically, as they were either too damaged to be identified (P-Pr-13-106) or were provided without identification by the New England Aquarium. Interestingly, one of the damaged positive nematodes (P-Pr-13-106) was collected from a phocid host presumed to be a grey seal (*H. grypus*). This seal (P-Pr-13-106) was originally by-caught in a fishing net off the coast of Cape Cod (exact location not recorded) and released overboard after being marked with an identification number (it is unknown if the seal was alive at the time of release). The carcass then washed up on a beach near Woods Hole, MA where it could not be formally re-identified by due to extensive decomposition other than by the original marking tag. Due to the presence of the original identifier, the stranded seal is presumed to be the same as the by caught seal, however due to the extensive decomposition this cannot be fully confirmed and no sample exists from the original seal to test. As such, our positive result for this nematode must be considered as potential evidence of seal heartworm infection in the grey seal rather than definitive proof. The eighth nematode was collected directly from the heart of seal during necropsy DO 5476 and has been previously identified by both DNA sequence and morphology (Keroack et al., 2016).

Infection by *A. spirocauda* in the grey seal has not been previously reported. Thus, this finding would represent the first evidence for *A. spirocauda* in the grey seal. Unfortunately, this parasite was damaged upon collection and could not be identified by morphology, and possible host misidentification cannot be fully ruled out. The ITS2 sequence of this parasite identified it as *A. spirocauda* at 99% identity/100% coverage respectively. Further, the SSU sequence showed strong similarity to the closely related parasite *A. viteae* (the only *Acanthocheilonema* species available for comparison prior to this study) showing 99% identity with 99% coverage: these values are consistent with other known *A. spircoauda* specimens from DO 5476 and P-Pr-13-104. These sequence results and the qPCR strongly suggest this parasite is seal heartworm, and represents the first potential evidence of infection in a grey seal.

3.6. Application of the C20 quantitative real-time PCR test to xenomonitoring

To test the potential of the assay to detect *A. spirocauda* in the proposed louse vector, *E. horridus*, 23 lice were screened for the presence of the parasite (Table 3). Individual lice were homogenized and DNA extracted for testing. Of these 23 lice, 2 were positive by the C20 diagnostic assay. The first positive louse was collected from a seal (NEAQ-14-048) an animal known to be infected by seal heartworm, as noted on the necropsy report and confirmed by our qPCR assay. The infection status of the seal (P-Pr-14-123) from which the second positive louse was collected, is unknown, and no nematodes from this seal were collected during the necropsy. The other 21 lice collected in this study were collected from seals where the infection status was not assessed, and thus may have been collected from uninfected seals. These results demonstrate that the assay can detect the parasite in the louse, and that the louse can be used as a first proxy to infer host infection status-although an infected louse may be found on an uninfected seal. Screening lice represents a less invasive method to monitor parasite epidemiology in various seal populations, and can be utilized as an alternative to blood collection as has been done with vectors of other important nematode infections (Higazi et al., 2011; Rao et al., 2016; Pilotte et al., 2017, 2016b).

Table 3
Xenomonitoring C20 qPCR results for louse isolates.

Louse specimen (host sp.)	Mean Ct value	SD
P-14-187 (*P.vitulina*)	nd[a]	nd
P-Pr-14-123 (*P. vitulina*)	15.72	0.10
NEAQ-14-048 (*P. vitulina*)	16.10	0.35
P-Pr-14-136 (*H. grypus*)	nd	nd
P-Pr-14-139 (*P. vitulina*)	nd	nd
P-14-178 (*P. vitulina*)	nd	nd
P-15-073 (*P. vitulina*)	nd	nd
P-Pr-14-032 (*H. grypus*)	nd	nd
P-Pr-11-020 (*H. grypus*)	nd	nd
P-Pr-13-056 (*H. grypus*)	nd	nd
P-Pr-14-132 (*H. grypus*)	nd	nd
P-Pr-14-136 (*H. grypus*)	nd	nd
P-Pr-14-138 (*H. grypus*)	nd	nd
P-Pr-13-139 (*P. vitulina*)	nd	nd
P-Pr-14-137 (*P. vitulina*)	nd	nd
P-Pr-12-069 (*P. vitulina*)	nd	nd
P-Pr-13-096 (*P. vitulina*)	nd	nd
P-Pr-14-059 (*P. vitulina*)	nd	nd
P-Pr-14-116 (*P. vitulina*)	nd	nd
P-Pr-14-140 (*P. vitulina*)	nd	nd
P-Pr-14-122 (*P. vitulina*)	nd	nd
P-Pr-14-164 (*P. virulina*)	nd	nd
P-Pr-14-119 (*P. vitulina*)	nd	nd
P-Pr-13-108 (A. spirocauda, *P. vitulina*, positive control)	19.95	0.05
O.circumlitus, negative control	nd	nd

[a] nd = not detected.

3.7. Potential use of the C20 qPCR as a sensitive diagnostic test for screening blood samples from seals

Uninfected seal blood samples spiked with *A. spirocauda* DNA were used to demonstrate that the assay can be used to reliably detect low levels of parasite DNA in seal blood (Table 4). Using a standard curve, we estimate the amount of parasite DNA in blood sample A is 0.0228 ng total and the amount in blood sample B is 0.0044 ng total (Fig. 2). While these samples do not represent true, naturally infected blood samples, they do show the potential of the assay to detect small quantities of *A. spirocauda* DNA in field samples. In known studies of prevalence of seal heart worm, the number of microfilariae (mf) per cc of blood is not reported, only quantitatively noted as mild, moderate, or severe (van den Broek, 1963; Claussen et al., 1991; Lunneryd, 1992; Measures et al., 1997; Lehnert et al., 2007, 2010, 2015). Only one study quantifies worm burden but reports only the number of adult worms found in parasitized seals, not mf in blood (Measures et al., 1997).

Table 4
Screening results for mock infected blood samples.

Sample	Total input DNA (ng)	Mean Ct value	SD
A: Spiked Blood 1 (Whole blood + 1X *A. spirocauda*)*	1	26.39	0.48
B: Spiked Blood 2 (Whole blood + 10X dilution *A. spirocauda*)*	1	30.43	1.26
C: Spiked Blood 3 (Whole blood + 100 X dilution *A. spirocauda*)*	1	nd[a]	nd
+ ctrl (P-Pr-13-104, *A. spirocauda*)	1	15.98	0.34
+ ctrl 10X dilution	0.1	23.92	0.07
+ ctrl 100X dilution	0.01	28.55	0.25
+ ctrl 1000X dilution	0.001	35.02	1.37
+ ctrl 10000X dilution	0.0001	38.57	0.02
+ ctrl 100000X dilution	0.00001	nd	nd

*Sample A, B, and C were generated by adding *A. spirocauda* DNA to whole seal blood to a final concentration of 0.05 ng/uL, 0.005 ng/uL, and 0.0005 ng/uL respectively. Total DNA from whole blood containing parasite DNA was then extracted as described in the methods.

[a] nd = not detected.

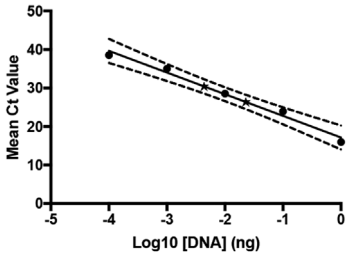

Fig. 2. Standard curve generated using the $\log_{10}$ of the ng of input *A. spirocauda* DNA plotted against Ct value. Unknown values are displayed as stars. For unknown samples, the total input DNA was 1 ng, which contains a mixture of seal DNA from the blood and *A. spirocauda* DNA. R^2 = 0.985 for linear fit of standards. Curve is described by the equation y = −5.63x + 17.16, where y is the log (ng) and x is the Ct value. 95% confidence intervals are denoted by the dotted lines.

Therefore, we could not mimic known natural infections in this spiking assay. Assuming a microfilaria has about 200 pg (0.2 ng) of DNA, our spiked assay is likely able to detect between ~0.1 and 0.2 mf/μl. Based on other reports of filarial infections, specifically reports of the heartworm *D. immitis,* this likely represents a low level, or very mild infection (Rojas et al., 2015). Based on these rough calculations, this test demonstrates the potential use of the C20 assay in detecting parasite DNA in seal blood from mildly to severely infected live seals. Further testing on blood samples collected from seals in rescue facilities will be done in the future, and any new clinical samples will have the number of microfilariae counted by microscopy.

4. Discussion

The generation of the C20 real-time PCR diagnostic assay represents a novel molecular tool for sensitively diagnosing seal heartworm. The utility of this approach for rapidly designing sensitive, species-specific and sensitive real-time PCR assays (Pilotte et al., 2016a), will allow for more comprehensive surveillance of *A. spirocauda* in seals. Traditionally, monitoring of seal heartworm has relied upon the collection of seal blood samples and the dissection of lice to detect the presence of filarial parasites using microscopic techniques (Measures et al., 1997; Lehnert et al., 2015). Recently, some advances have been made in the application of molecular tools, including use of the mitochondrial cytochrome oxidase subunit 1 (COI) gene as a means of identification (Lehnert et al., 2015). The method of developing a specific quantitative real-time PCR assay has numerous advantages over the use of morphology or single-gene barcodes alone. Morphology, while incredibly useful and informative, requires expert analysis, and is often not feasible for labs without such experts (Blaxter, 2004; Moritz and Cicero, 2004; Waugh, 2007; Packer et al., 2009). The pipeline used in this study will be readily translatable to other pathogens, as it was pioneered for use in other nematode parasites (Pilotte et al., 2016a). Further, while development of these types of assays do require sequencing equipment, once generated they are easy amenable to diagnostic laboratories. With the advent of portable PCR and qPCR machines, assays such as this may soon be feasible to perform in the field. Additionally, parasites are often obtained in a damaged/degraded state that makes morphological analysis impossible.

Similarly, the use of single-gene barcodes, while originally hailed as the gold standard of species identification, has been found to be fraught with difficulties (Moritz and Cicero, 2004; Will and Rubinoff, 2004; Will et al., 2005; Waugh, 2007; Borisenko et al., 2009). The lack of publicly available DNA sequence data for many parasite species to use as a reference database accounts for some of the difficulty encountered when using single gene barcodes. Accurate barcoding relies on the existence of such a comprehensive reference database. This database needs to be generated from sequences that are linked to reliable

voucher specimens (Borisenko et al., 2009). To generate a comprehensive database, sampling of representative organisms must be massive (Ferri et al., 2009). A real-time qPCR assay based on detecting highly repetitive genomic repeats results in a test that is both extremely species-specific and sensitive, ultimately improving methods for parasite identification in clinical samples from live seals or in seal lice, even if the samples are obtained in poor condition. This assay provides non-invasive modalities for sampling from live seals, either in rehabilitation or as part of prevalence surveys. Further, sensitive diagnostic tools will be essential in monitoring diseases in endangered animals where large-scale sampling is impossible.

The C20 real-time qPCR is the first specific diagnostic test available for molecular xenomonitoring to screen lice. As a model, extensive use has been made of molecular xenomonitoring to screen other insect vectors to estimate the infection levels in other systems (Pilotte et al., 2017). The ability to monitor seal heartworm via screening lice offers a similar model system for estimating prevalence. Additionally, this assay can reliably detect parasite DNA from single worms and from parasite DNA in seal blood, demonstrating its high sensitivity. This diagnostic tool can be used in future studies to examine the prevalence of *A. spirocauda* in seal populations by screening blood samples or lice collected from live animals. This tool can also be used to identify nematodes that are collected from decomposed hosts during autopsy, or specimens damaged during collection or storage which cannot otherwise be identified.

Previously, infection by *A. spirocauda* in grey seals (*H. grypus*) has not been reported. Using the C20 quantitative real-time PCR assay developed in this study, we present potential evidence of the first infection of seal heartworm in the grey seal. Such a finding warrants follow up studies in larger grey seal populations. The parasite has been previously found in harbor seals (*Phoca vitulina*), ringed seals (*Phoca hispida*), harp seals (*Phoca groenlandica*), hooded seals (*Cytsophora cristata*), and potentially fur seals (*Callorhinus ursinus*) (Measures et al., 1997; Leidenberger et al., 2007; Felix, 2013). The absence of seal heartworm in grey seals has been attributed to either insufficient sampling, resistance to infection, or extreme mortality from infection. The first explanation seems the most likely, and further sampling is needed (Measures et al., 1997). It has been shown that grey seals are parasitized by the seal louse, the proposed vector of the parasite, so infection by seal heartworm in grey seals may be possible (Lehnert et al., 2015). It is also possible that parasitemia in infected grey seals is low enough that it cannot be detected using other molecular techniques, whereas this sensitive real-time PCR assay can detect extremely low concentrations of filarial DNA.

Prevalence of seal heartworm, *A. spirocauda*, has been reported to be increasing, thus the possible spread of the parasite into grey seals provides justification for increased monitoring of phocid seal populations to track the spread of disease (Lehnert et al., 2015). Furthermore, the parasite has recently been reported in the highly endangered monk seal population, further stressing the need to accurately monitor the range of this parasite (Papadopoulos et al., 2010). The C20 diagnostic test developed here provides an important tool for monitoring the spread of the disease into new hosts and geographical ranges. The ease and speed of generation of NCR based diagnostics can be used in the future to generate novel tools for other marine mammal pathogens of interest.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2018.04.001.

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