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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